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13. ABSTRACT *(Maximum 200 Words)*

Studies with gene knockout mice have demonstrated that M6P/IGF2R is an inhibitor of IGF2-dependent embryonic growth. Tumor-associated M6P/IGF2R mutations that may disrupt IGF2 binding have been described suggesting that the IGF2 antagonist action of M6P/IGF2R may also play a role in suppressing tumorigenesis. To investigate the role of M6P/IGF2R as an IGF2 antagonist in breast cancer, M6P/IGF2R was overexpressed in MCF7 cells. Despite overexpression of wildtype or binding defective mutant M6P/IGF2R, IGF2-dependent growth in either monolayer or in suspension culture was unaffected. MCF7 cell clones were also engineered to inducibly overexpress wildtype M6P/IGF2R (iwt1 cells). Despite 10-fold induction of M6P/IGF2R expression, IGF2-dependent growth and insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation were unaltered when compared with control cells. We had previously demonstrated that affinity of IGF2 for M6P/IGF2R suppresses autocrine IGF2 activity. These data led us to hypothesize that M6P/IGF2R may operate to inhibit autocrine IGF2 but not efficiently suppress exogenous IGF2 action. We therefore transfected iwt1 cells to constitutively express IGF2. We were unable to detect any effect of M6P/IGF2R overexpression on apoptosis or IRS-1 phosphorylation signaling but could demonstrate antagonism of autocrine IGF2-dependent growth in monolayer culture. Although the functional evidence is insufficient to make the case that M6P/IGF2R is a tumor suppressor, the genetic evidence is compelling and justifies further experiments in various model systems.

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I would first like to draw attention to the fact that the project discussed in this report is indeed different from the one originally proposed, entitiled "IGF regulation of cell adhesion in breast cancer". However, in my Annual Report for June 1997 through July 1998, I addressed the changes in the project and also submitted a revised Statement of Work which was approved in the Review of the 1997-1998 Report. The following Final Report below discusses the approved revised investigation where the focus of the project was to provide functional data to support recent genetic evidence that mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) may be a tumor suppressor in breast cancer.

INTRODUCTION

M6P/IGF2R is a multifunctional transport protein. Established physiological functions of M6P/IGF2R include: (1) to facilitate the transport of newly synthesized mannose 6-phosphorylated (M6P) lysosomal proteins from the Golgi to early lysosomal compartments (1); (2) to facilitate endocytosis of extracellular M6P-tagged lysosomal enzymes (2); (3) to facilitate proteolytic activation of transforming growth factor β (TGFβ), a negative growth regulator of epithelial cells (3); and (4) to target insulin-like growth factor 2 (IGF2) for lysosomal degradation, thereby preventing insulin-like growth factor 1 receptor (IGF1R) activation (4;5). It has been hypothesized that loss, or aberrant expression of M6P/IGF2R will contribute to tumor cell growth by decreasing the growth inhibitory effect of TGFβ, by promoting tumor invasion through lysosomal protein misrouting and by increasing the mitogenic and cell survival effect of IGF2 (6).

Molecular cloning studies and transgenic mouse studies have provided some evidence that M6P/IGF2R plays a role in negative growth regulation. Genetic studies have identified M6P/IGF2R as a putative tumor suppressor. The gene locus at 6q25-26 is reportedly subject to loss of heterozygosity (LOH) accompanied by loss-of-function point mutations in the remaining allele in both breast and liver carcinomas (7;8). Furthermore, somatic mutations in coding region microsatellites of *M6P/IGF2R* have been documented in a variety of replication error positive (RER+) tumors of the gastrointestinal tract, brain and endometrium (9-12).

Gene knockout studies in mice suggest that growth suppressive effects of M6P/IGF2R is due to antagonism of IGF2-dependent growth. M6P/IGF2R knockout mice die *in utero* due to cardiac overgrowth. The M6P/IGF2R null fetuses have a lysosomal enzyme storage defect, elevated levels of IGF2 and are approximately 30% larger than normal littermates (13;14). This overgrowth phenotype is reversed in the IGF2 null background although the lysosomal enzyme storage is still deficient (15).

Although the genetic evidence is compelling, there is little functional evidence that supports the theory that M6P/IGF2R is a *bona fide* tumor suppressor. We hypothesized that the **M6P/IGF2R is a tumor suppressor in IGF2-sensitive malignancies**. To this end, the following aims were identified:

1. To investigate the effect of overexpression of M6P/IGF2R on exogenous IGF2 dependent growth *in vitro* utilizing ligand-selective receptor constructs.
2. To determine whether M6P/IGF2R overexpression affected autocrine IGF2 dependent growth and survival *in vitro*.

The fulfillment of these aims in relation to the support or rejection of our proposed hypothesis is presented and discussed in this Final Report.

BODY

To identify the potential role of M6P/IGF2R in growth regulation, mutations were introduced in the receptor which selectively disrupt either IGF2 or M6P-binding. IGF2*def*R, deficient in IGF2 binding, and M6P*def*R, deficient in M6P binding, in addition to an inactive frameshift negative control D95Stop were sublconed into the pcDNA3 expression vector and used in transfection studies. To further support our results, we employed the Tet-On system in MCF7 cells to investigate the effect of M6P/IGF2R overexpression when induced by doxycyline treatment. We hypothesized that increasing the M6P/IGF2R expression by transfection would result in M6P/IGF2R-mediated growth suppression by antagonizing IGF2-dependent proliferation. Our data reveal that overexpression of M6P/IGF2R does not regulate exogenous IGF2-dependent growth of the model system used in these studies.

Statement of Work proposed for Months 1 to 12

1. Transfect MCF7 cell line with four bovine M6P/IGF2R expression vectors containing wildtype and mutant cDNAs.
2. Investigate the response to IGF2 in MCF7 cells overexpressing wildtype and mutant M6P/IGF2R receptors.
3. Develop an inducible expression system for bovine M6P/IGF2R in MCF7 cells.
4. Clone the human M6P/IGF2R cDNA into a constitutive expression vector and express M6P/IGF2R in receptor null mouse D9 cells.

<u>Ligand-binding defective M6P/IGF2R mutants</u>

For the experiments in this study, we employed bovine M6P/IGF2R cDNA to generate selective ligand binding mutant constructs. The bovine M6P/IGF2R cDNA was utilized because it is well characterized, and in transfection studies with human cell lines, transfected bovine receptor can be distinguished form endogenous human M6P/IGF2R with species-specific monoclonal antibodies. Further, the human and bovine M6P/IGF2R cDNA constructs share greater than 80% sequence homology

(16) and experiments examining the mannose 6-phosphate (M6P) protein sorting properties of the human and bovine M6P/IGF2R demonstrated that there were no functional differences between the two M6P/IGF2R species (17;18).

Wildtype bovine M6P/IGF2R (wt) and M6P-binding deficient bovine M6P/IGF2R (M6P*def*R) cDNAs were kindly provided by Dr. N. Dahms (19;20). M6P*def*R was generated by a dual combination of arginine to alanine substitutions at amino acid positions 435 and 1334 which effectively disrupted M6P interactions (Figure 1). An IGF2-binding deficient bovine M6P/IGF2R (IGF2*def*R) was generated in the Ellis laboratory (Figure 1) by a site-directed mutagenesis polymerase chain reaction approach (Schumaker and Ellis, unpublished). This binding mutant was modeled from an IGF2-binding defective recombinant human M6P/IGF2R fragment with an isoleucine to threonine substitution described by Garmroudi et al. (21). A homologous substitution was made in the bovine construct at position 1581 to generate the IGF2*def*R construct.

As a negative control, a frame shift mutation encoding a nonfunctional truncated bovine M6P/IGF2R (D95Stop) was also synthesized (Schumaker and Ellis, unpublished). The nucleotide sequence of the receptor constructs was confirmed by sequencing and then subcloned into a constitutive expression vector, pcDNA3, for transfection.

IGF2 and M6P binding characteristics of wt and mutant M6P/IGF2R

To determine the affinity of the various receptor constructs for IGF2, expression vectors encoding the receptor constructs were transfected into M6P/IGF2-deficient L cells (clone D9) (22). Transfected D9 cells were selected with G418 for three weeks and pooled. To assess IGF2 binding of the various receptor constructs, membrane preparations of D9 cells transfected with the various receptor constructs were isolated for radioligand binding assays. Membrane preparations were dried onto 96-well dishes, allowed to bind ^{125}I-IGF2 with various concentrations of competing unlabelled IGF2, washed, and the amount of bound radiolabelled IGF2 was determined with a gamma counter. This assay demonstrates that the negative control mutant receptor, D95Stop, and the mutant receptor deficient for IGF2-binding, IGF2*def*R, were unable to significantly bind IGF2. In contrast, the wildtype receptor construct and the M6P-binding deficient mutant receptor, M6P*def*R bind IGF2 with similar affinity (Figure 2). To further assess IGF2 binding, Lisa Schumaker in the Ellis lab performed ^{125}I-IGF2 cross-linking assays. We were unable to detect any ^{125}I-IGF2 cross-linking of the negative control mutant D95Stop. IGF2*def*R, the mutant engineered for deficient IGF2-binding, had the same cross-linking profile as the negative control. Conversely, wt M6P/IGF2R and the M6P-binding deficient mutant M6P*def*R efficiently crosslinked to

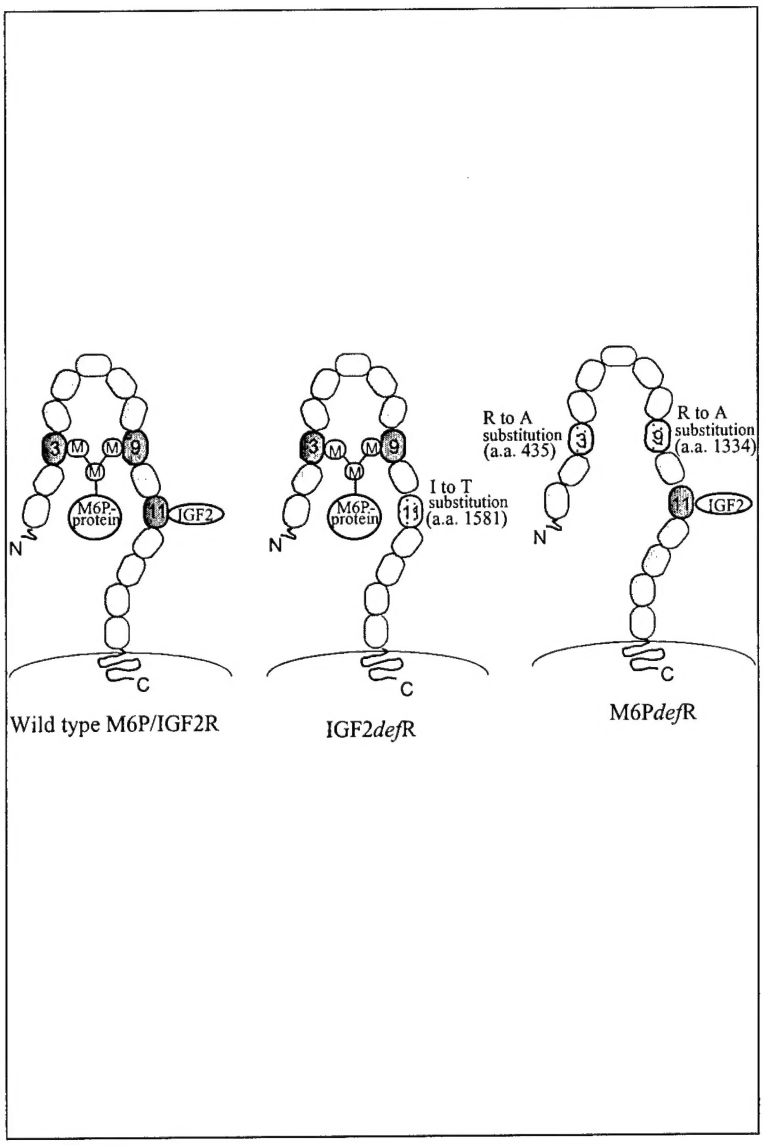

Figure 1. Site directed mutant constructs of M6P/IGF2R. Bovine wildtype M6P/IGF2R (wt) and selective ligand binding mutant receptor constructs were generated. M6P*def*R incorporates dual arginine to alanine substitutions that abolishes M6P protein binding. IGF2*def*R was generated with an isoleucine to threonine point mutation at amino acid position 1581. In addition, a frameshift mutation encoding a non-functional truncated M6P/IGF2R was generated as a negative control (D95Stop).

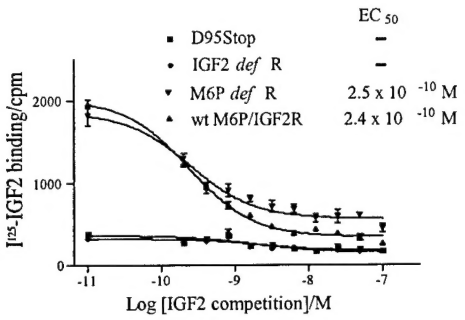

Figure 2. IGF2 binding to wildtype and mutant receptors. B, analysis of radioligand binding assay. ^{125}I-IGF2 was allowed to bind to microsomal membrane preparations of D9 cells transfected with the various receptor constructs and subjected to various concentrations of unlabelled competing IGF2. Bound radiolabeled IGF2 was measured by an automated gamma counter.

^{125}I-IGF2; this crosslinking could be suppressed with unlabelled IGF2 competition but not unlabelled IGF1 competition.

To estimate mannose 6-phosphate binding capacity, proteins were extracted from transfected D9 cell microsomal membranes and allowed to bind pentamannosyl 6-phosphate-conjugated (pM6P) sepharose beads (These M6P binding experiments were also completed by Lisa Schumaker in the Ellis lab). Following incubation with membrane extracts, successive elutions were collected as the beads were washed first with binding buffer, then with buffer containing 10 mM glucose 6-phosphate, and finally with buffer containing 10 mM mannose 6-phosphate. M6P elution was repeated until all elutable M6P/IGF2R was removed from the beads. In these experiments, the ability of IGF2*def* R to bind to pM6P beads was indistinguishable from wt M6P/IGF2R. We therefore concluded that the I1581T substitution did not significantly alter interactions with mannose 6-phosphate. The capacity of M6P*def* R to bind to pM6P was reduced to approximately 5% of wt M6P/IGF2R and IGF2*def* R.

Cellular proliferation of D9 cells is not affected by expression of wildtype or mutant M6P/IGF2R.

We first examined the effects of M6P/IGF2R expression in D9 cells. The null background of D9 cells makes them an attractive model in which to study the effect of M6P/IGF2R overexpression. These cells have been highly utilized by other investigators to study the trafficking properties of M6P/IGF2R, although effects on growth have not been described (23;24).

The *in vitro* growth of wild-type and mutant M6P/IGF2R transfected D9 cells was compared with control (D95Stop) transfected cells by employing a WST1 colorimetric cell proliferation assay. Growth rates were unaffected in M6P/IGF2R transfected D9 cells in serum free media, in 5% serum or in the presence of various IGF1 or IGF2 concentrations. During the course of these experiments, it was determined that D9 cells were insensitive to IGF1 and IGF2 treatment (Figure 3). The lack of effect of M6P/IGF2R expression could be explained by the fact that growth of the D9 cells was not IGF2-dependent. We therefore decided to investigate the hypothesis in a breast cancer cell line that not only had a null M6P/IGF2R background, but also was sensitive to IGF1 and IGF2. Western blot and IGF2 cross-linking assays were performed to identify one such cell type, but none was identified (Schumaker and Ellis, unpublished). We therefore chose MCF7 to conduct our experiments since this cell line had a low level of endogenous M6P/IGF2R compared to the other cell lines investigated.

Appropriate subcellular distribution of exogenous M6P/IGF2R in MCF7 cells

MCF7 cells were transfected to constitutively express either wt M6P/IGF2R or each of the mutant receptor constructs. To determine that exogenous M6P/IGF2R in MCF7 cells was appropriately localized

in the Golgi and endosomal compartments, the transfected cells were grown on microscope slides, fixed, permeablized and probed with a polyclonal antibody to M6P/IGF2R. The stained cells were viewed by confocal microscopy (Figure 4). MCF7 cells transfected with the negative control D95Stop exhibited extremely low levels of M6P/IGF2R, indicating the low background of endogenous human M6P/IGF2R in MCF7 cells. The pattern of staining for wt M6P/IGF2R, M6P*def*R and IGF2*def*R constructs were identical and consistent with M6P/IGF2R localization at the Golgi and endosomal compartments. This distribution of M6P/IGF2R is indistinguishable from that of human wildtype M6P/IGF2R (hM6P/IGF2R) overexpressed in MCF7 cells or bovine wildtype M6P/IGF2R overexpressed in the murine fibroblast-like D9 cell line. Furthermore, the pattern of exogenous M6P/IGF2R localization shown here is similar to that observed in PD3881 cells transfected to overexpress human M6P/IGF2R, as well as in normal rat fetal cardiac myocytes (25). We therefore concluded that the distribution of wildtype and mutant M6P/IGF2R is appropriate in the transfected cells.

Constitutive M6P/IGF2R overexpression does not affect proliferation of MCF7 cells

Transfected cells expressed a 5-fold increase (determined by densitometry not shown) of M6P/IGF2R over endogenous levels (Figure 5A). Western blot analysis with a bovine receptor specific monoclonal antibody and with a polyclonal antibody to M6P/IGF2R confirmed that transfected wt MCF7/IGF2R, IGF2*def*R and M6P*def*R were expressed at similar levels in each pool. We studied the effect of M6P/IGF2R overexpression on proliferation in low serum (0.1% FBS) medium supplemented with either 10nM IGF1 or IGF2. Figure 5, panels B and C, present the compilation of four separate WST-1 proliferation assays. As expected IGF1-dependent growth was similar for all the transfectants (Figure 5B) since IGF1 has no affinity for M6P/IGF2R. However, it was surprising to find that overexpression of M6P/IGF2R did not affect the IGF2-dependent growth rate of cells overexpressing wildtype or either of the two mutant receptors when compared to cells transfected with the negative control (Figure 5C).

We also investigated the consequence of M6P/IGF2R overexpression on the anchorage-independent growth capability of cells. To control for any differences in cell plating among the four pooled cell lines in the soft agar assays, we have presented our results as the ratio of the number of colonies (greater than 60 μm) formed from stimulation with 10 nM IGF1 to the number of colonies formed from stimulation with 10 nM IGF2. If overexpression of M6P/IGF2R negatively affects IGF2- but not IGF1-dependent growth of MCF7 cells as hypothesized, cells overexpressing wt M6P/IGF2R and M6P*def*R would form less colonies in response to IGF2 then cells expressing IGF2*def*R or the negative control. Thus, the ratio of IGF1-dependent growth:IGF2-dependent growth would be greater for those cells expressing wt or M6P*def*R compared to that of control cells. However, we did not detect a

difference in the number of colonies from cells overexpressing the various receptor constructs; the IGF1:IGF2 ratio of colonies formed in soft agar after a 2 week period from cells expressing either wt M6P/IGF2R or M6P*def*R was not significantly different from cells expressing IGF2*def*R or the negative control (Figure 5D).

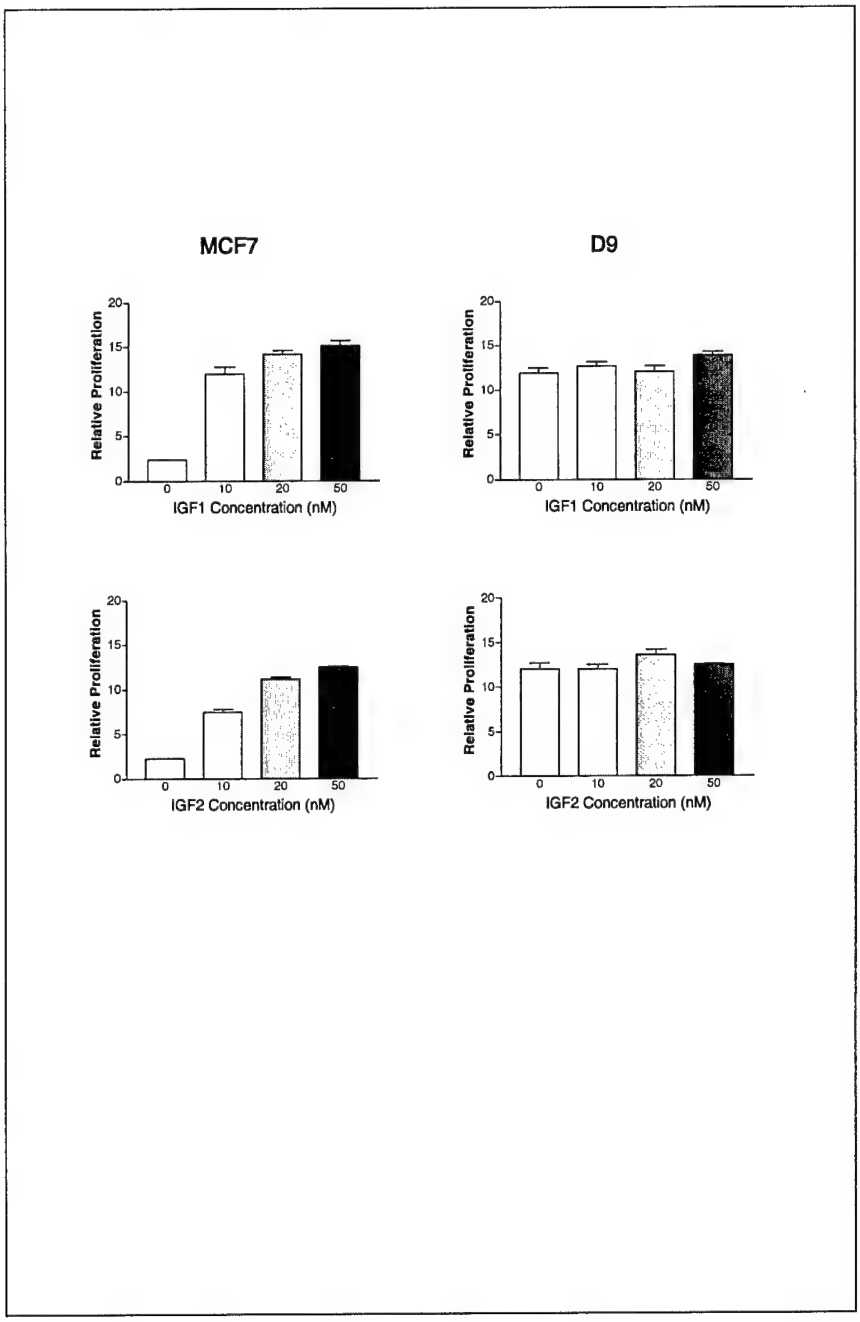

Figure 3. Growth of D9 cells is not IGF dependent. D9 cells and MCF7 cells were plated in 96 well dishes in triplicate and treated with various concentrations of IGF1 and IGF2. Unlike MCF7 cells that respond to IGFs in a dose dependent manner, D9 cells are unresponsive to IGF1 or IGF2 stimulus.

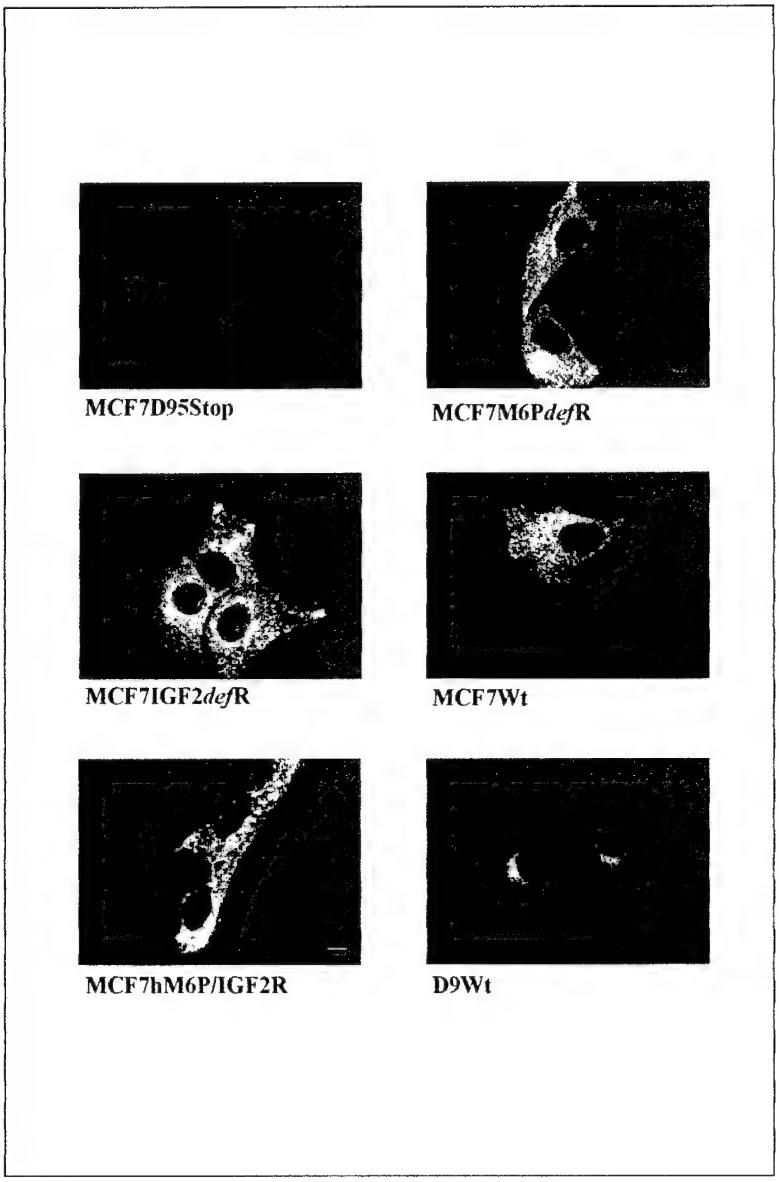

Figure 4. Subcellular localization of M6P/IGF2R in transfected MCF7 and D9 cells. MCF7 cells expressing bovine M6P/IGF2R receptor constructs, D95Stop, IGF2*def*R, M6P*def*R or wt M6P/IGF2R, were cultured on glass coverslips, fixed and immunostained with an M6P/IGF2R polyclonal antibody and fluorescein-conjugted secondary antibody. MCF7 cells expressing human M6P/IGF2R (hM6P/IGF2R) and D9 cells expressing bovine wt M6P/IGF2R were also stained for positive controls. 600x or 1000x magnification, the bar represents 10 μm.

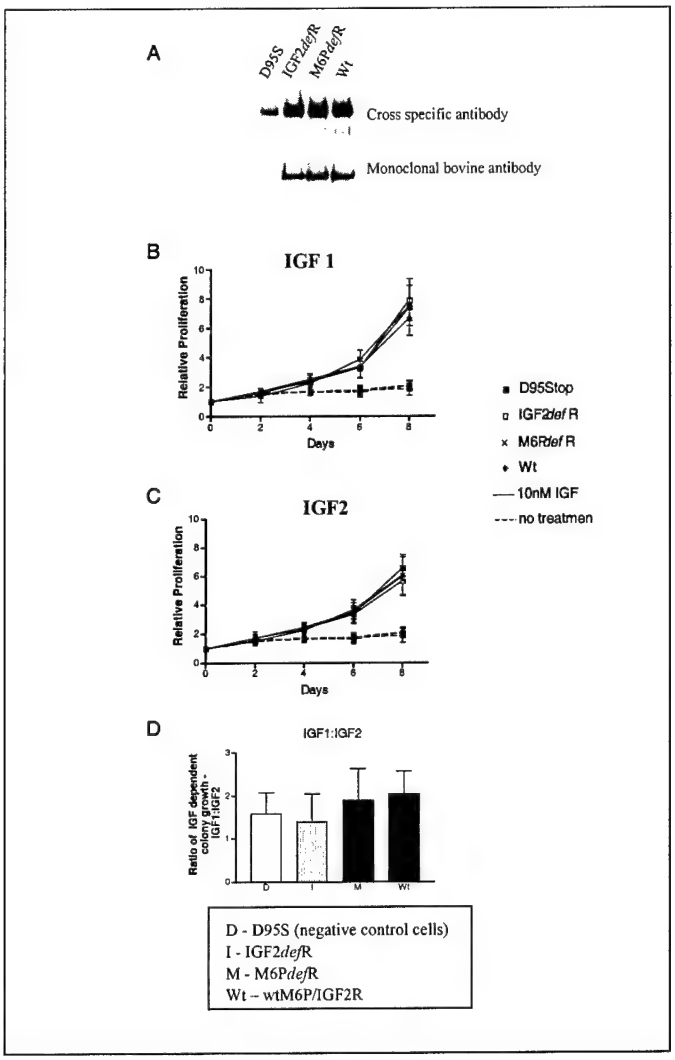

Figure 5. Relative proliferation of MCF7 cells constitutively expressing D95Stop, IGF2*def*R, M6P*def*R or wt M6P/IGF2R. A, to verify expression of the respective receptors, transfected MCF7 lysates were screened by Western blotting. Upper panel shows blot probed with a species cross-specific polyclonal antibody; lower panel shows blot probed with a bovine specific monoclonal antibody. **B** and **C**, relative proliferation of cells expressing the various M6P/IGF2R constructs was determined by WST-1 readings at the indicated time points for cells treated at day 0 with a single dose of 10nM IGF1 or 10nM IGF2. Values are an average of 4 independent experiments and the error bars indicate the SEM. **D**, Cells plated in soft agar were treated with 10nM IGF1 or 10nM IGF2 and the number of colonies counted after 14 days. The ratio of the number of colonies in IGF1 to the number of colonies in IGF2 was analyzed. The bar chart represents the average of the ratios from 6 independent experiments and error bars indicate the SEM.

Statement of Work proposed for Months 13 to 24

1. Establish the growth effects of inducible expression of bovine M6P/IGF2R in MCF7 cells in vitro and investigate these growth effects of ligand binding mutants already established by the laboratory.
2. Develop an animal protocol for the investigation of the phenotypic consequences of M6P/IGF2R in vivo in tumor xenografts. Conduct pilot experiments.
3. Subclone the human M6P/IGF2R into a tetracycline inducible expression vector and compare growth effects of human and bovine M6P/IGF2R in vitro.

Inducible overexpression of wt M6P/IGF2R in MCF7 cells does not alter proliferation

The findings with pooled transfected MCF7 cells constitutively expressing M6P/IGF2R did not support our original hypothesis that the IGF2-antagonist action of M6P/IGF2R has a role in tumor suppression of IGF2 sensitive malignancies. To confirm this negative result, we investigated this question using a system that allows for inducible expression of M6P/IGF2R. MCF7 cells previously transfected with a response plasmid for the inducible Tet-On system (made available by Dr. A. Lee, University of Texas Health Center, and Dr. D. Yee, University of Minnesota Cancer Center) were transfected to overexpress M6P/IGF2R under the control of a doxycycline inducible promoter. Several clones were isolated and screened for doxycycline inducible expression of the wt M6P/IGF2R. A clonal population, iwt1, was identified and expanded for further study. A 10-fold induction of M6P/IGF2R expression (determined by densitometry, not shown) could be detected after treatment with 1 μg/ml doxycycline in 10% FBS for 24 hours (Figure 6Aand B). Western blot analysis also shows induction of M6P/IGF2R from 6 hours to a maximal level at 48 hours which is maintained even at 96 hours with either 250 ng/ml or 500 ng/ml of doxycyline treatment (Figure 6C and D). In further experiments, 250 ng/ml doxycyline was used to induce M6P/IGF2R expression.

Confocal microscopy, shown in Figure 7, confirmed that the subcellular distribution of doxycycline induced wt M6P/IGF2R in iwt1 was identical to that shown in MCF7 pools constitutively overexpressing the receptor. Examination of immunofluorescent cells also demonstrated that approximately 30% to 40% of cells inducibly overexpressed the receptor when treated with doxycycline.

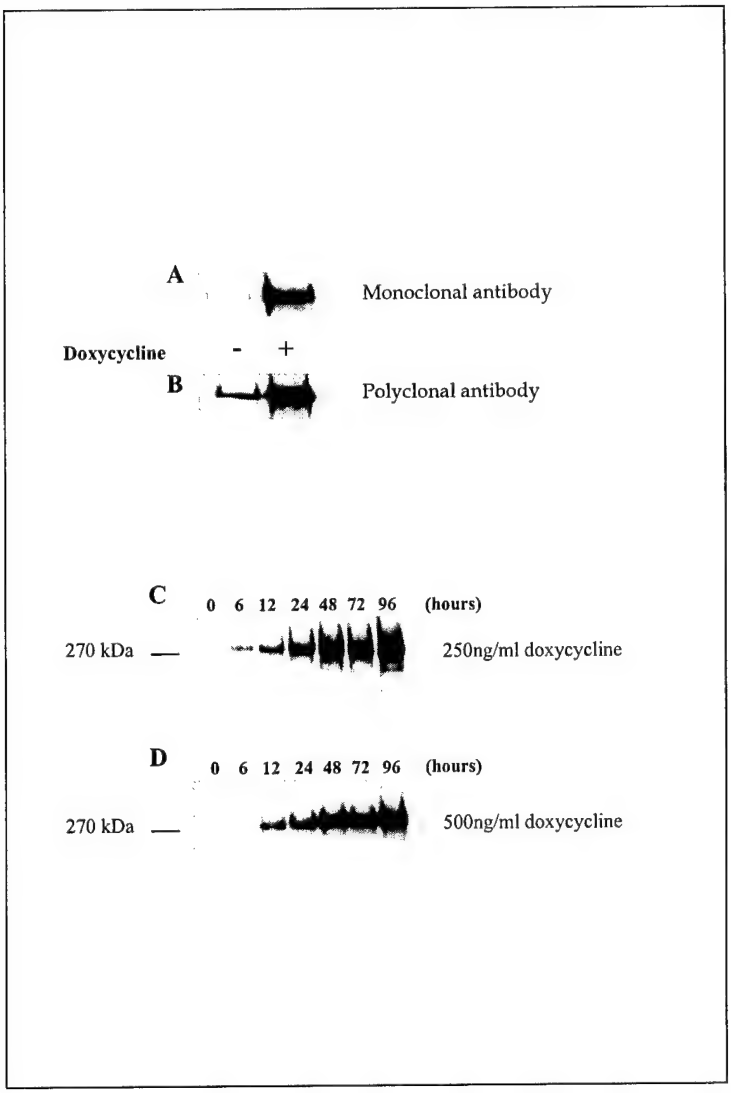

Figure 6. Inducible expression of bovine wt M6P/IGF2R in MCF7 cells (iwt1 cells). **A**, western blot analysis of whole cell lysates of MCF7 cells treated with or without 1 μg/ml doxycycline for 24 hours demonstrating inducible expression of wt M6P/IGF2R. The blot was probed with a monoclonal antibody for M6P/IGF2R and stripped and reprobed with a polyclonal antibody to demonstrate endogenous and exogenous M6P/IGF2R expression. **B**, western blot analysis showing a time course of inducible M6P/IGF2R expression in cells treated with either 250ng/ml or 500ng/ml. The blot was probed with polyclonal antibody to M6P/IGF2R.

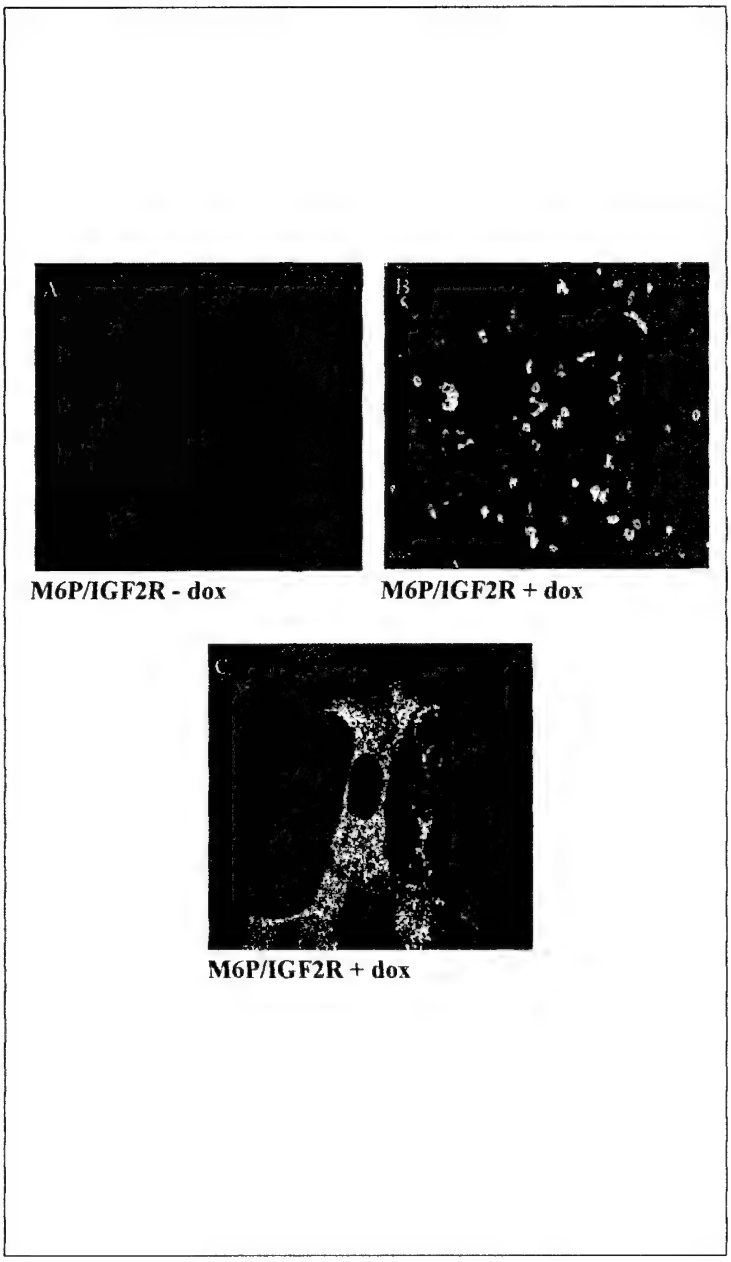

Figure 7. Subcellular localization of M6P/IGF2R in iwt1 cells. iwt1cells were cultured on glass coverslips and treated **A**, without doxycycline or **B** and **C**, with 1 μg/ml doxycycline for 24 hours. The cells were then fixed, permeablized and immunostained with an M6P/IGF2R polyclonal antibody and fluorescein-conjugated secondary antibody. Subcellular localization of the induced receptor is similar to what has been published. 200X or 600X magnification.

With this tightly regulated inducible expression model, we repeated WST-1 proliferation assays and studied the effect of induced overexpressed M6P/IGF2R on the growth rate of cells (treated with doxycycline) compared to control cells (not treated with doxycyline) in response to IGF1 and IGF2. iwt1 cells were plated in 96-well dishes and treated with or without doxycycline to induce M6P/IGF2R expression for 24 hours before IGFs were added. It must be noted that 40nM IGF concentration was used in these assays, compared to 10nM IGFs with the MCF7 cells that constitutively expressed the transfected receptor constructs, because the inducible cells did not respond well to lower concentrations of IGFs. Figure 8A and 8B demonstrate that overexpression of wt M6P/IGF2R did not affect response to either IGF1 or IGF2.

Anchorage independent growth was also unaffected by overexpression of M6P/IGF2R. iwt1 cells treated with doxycycline to induce M6P/IGF2R overexpression showed no change in colony growth compared to untreated cells. The ratio of IGF1:IGF2 dependent number of colonies (greater than 60 μm) was similar whether or not cells were induced to overexpress M6P/IGF2R (Figure 8C).

IRS-1 stimulation in response to IGF2 is unaffected by M6P/IGF2R overexpression

IGF2 evokes cellular proliferation and anti-apoptotic activity through IGF1R-mediated activation of downstream signaling molecules including insulin receptor substrate-1 (IRS-1) which is phosphorylated on multiple tyrosine residues in response to IGF1R activation (26). Our original hypothesis predicted that overexpression of wt M6P/IGF2R would increase IGF2 degradation resulting in reduced IGF1R activation and IRS-1 phosphorylation in response to IGF2 stimulation, but not IGF1 stimulation. Therefore, in addition to cellular proliferation, we investigated the consequence of M6P/IGF2R overexpression on the kinetics of IGF-induced IRS-1 tyrosine phosphorylation.

iwt1 cells were plated in 6cm dishes and treated with or without doxycycline in growth media for 48 hours. After serum starvation for 24 hours, the cells were treated with or without IGFs, lysed and cell extracts subjected to immunoprecipitation (IP) with anti-IRS-1 antibody followed by Western blotting with an anti-phosphotyrosine monoclonal anitbody. Figure 9A is an IP/Western blot of uninduced iWt1 treated with various concentrations of IGF2 for 5 minutes. An increase in IGF2-dependent IRS-1 phosphorylation could be detected with 30 nM IGF2 and this increased with increasing IGF2 concentration. Therefore, we chose to use 40 nM IGF concentrations in all IP experiments. Figure 9 is representative of 3 IRS-1 IP experiments demonstrating no reduction of IRS-1 activation in cells overexpressing wt M6P/IGF2R in response to either IGF2 or IGF1 treatment (Figure 9B and C) for 1 hour. Figure 9D is a Western blot for M6P/IGF2R demonstrating that overexpression of the receptor was induced with doxycyline treatment.

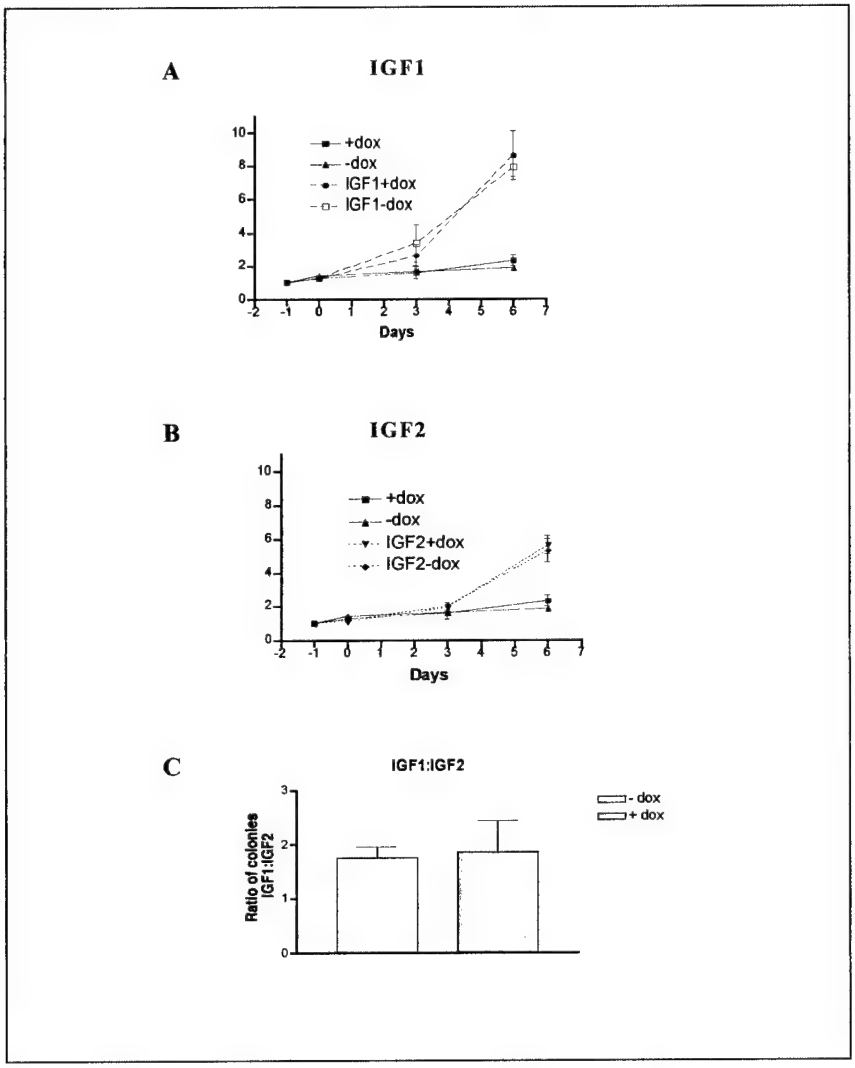

Figure 8. Overexpression of M6P/IGF2R does not affect proliferation of iwt1 cells. **A** and **B**, cells treated without or with doxycycline to induce wt M6P/IGF2R expression were given no treatment or treated with 40nM IGF1 or IGF2. Proliferation was determined by WST-1 at the times indicated. The graphs are representative of 3 experiments of data points in triplicate with iwt1 cells and the error bars represent SEM. **C,** Cells plated in soft agar were treated without or with doxycycline for 24 hours then treated with 40nM IGF1 or 40nM IGF2. The number of colonies was counted after 14 days and the ratio of the number of colonies in IGF1 to the number of colonies in IGF2 was determined for cells treated without or with doxycycline to inducibly express wt M6P/IGF2R.

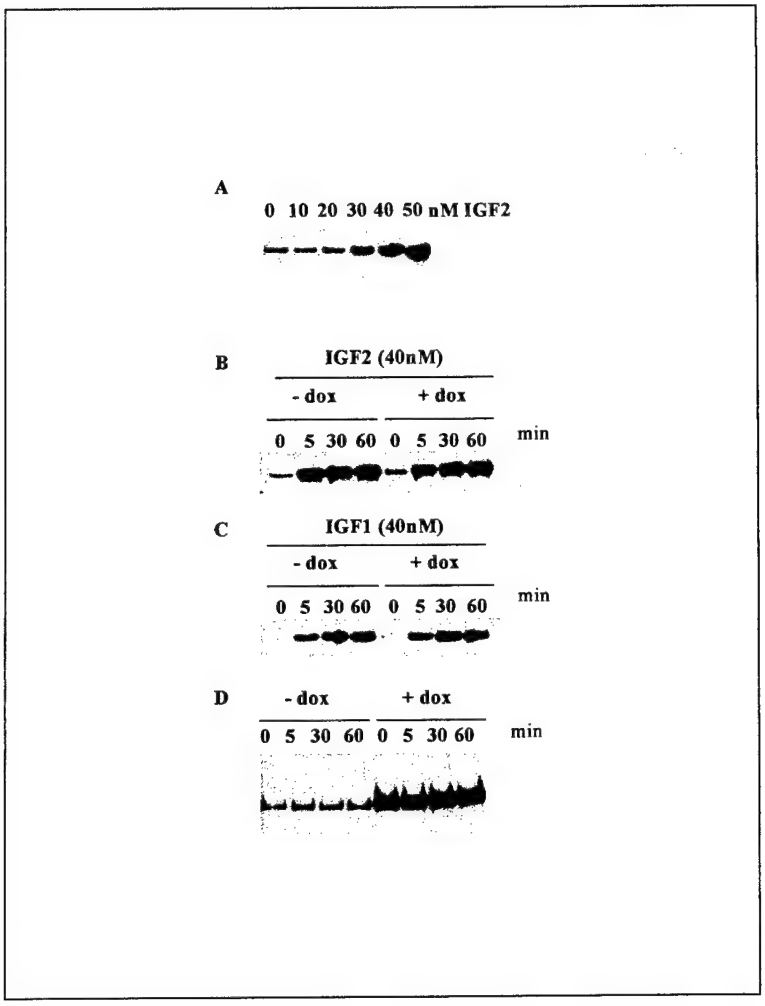

Figure 9. IRS-1 Phosphorylation Kinetics. Lysates from serum-starved cells stimulated with IGF1 or IGF2 for the times indicated were immunoprecipitated with an IRS-1 antibody and immunoblotted with a phosphotyrosine antibody. **A**, a dose response IRS-1 IP was done to determine the optimum concentration of IGFs to use in experiments. **B** and **C**, a representative gel of three independent experiments is shown. Cells were treated with 40nM IGFs and without or with 1 μg/ml doxycycline to induce M6P/IGF2R expression. **D**, western blot of pre-immunoprecipitated lysates of cells treated without or with doxycycline to induce wt M6P/IGF2R expression and probed with a polyclonal antibody to the M6P/IGF2R.

This finding that M6P/IGF2R overexpression does not affect IGF2-induced IRS-1 activation supports the results of the growth experiments, where M6P/IGF2R overexpression did not result in any change in IGF2-dependent proliferation.

Although the statement of work for Months 13 to 24 proposed animal experiments, before attempting these expensive experiments, in the light of our negative data, we decided to first investigate whether the overexpressed M6P/IGF2R was indeed functional in our model system. We therefore performed experiments to investigate some trafficking properties of the overexpressed M6P/IGF2R and looked at cathepsin D secretion and IGF2 internalization. The negative results determined in this project to this point and the attempt to determine functionality of the model system used engaged the time during months 25 to 36 rather than what was proposed in the SOW.

Expression of M6P/IGF2R does not alter cathepsin D secretion

As previously discussed, a major function of M6P/IGF2R is to mediate transport of newly synthesized lysosomal enzymes. Although 5 to 10% of the receptor pool cycles to and from the plasma membrane (27;28), most of the M6P/IGF2R functions intracellularly, transporting newly synthesized mannose 6-phosphorylated proteins from the Golgi to early endosomes.

MCF7 cells have been shown to produce and secrete large amounts of cathepsin D. To address functionality of the overexpressed M6P/IGF2R in our system, we examined cathepsin D trafficking in our cells. We hypothesized that increased receptor expression would alter cathepsin D secretion, most likely due to more efficient targeting to lysosomal compartments via M6P/IGF2R trafficking. Figure 10 shows western blot analysis of conditioned media from cells treated without or with doxycycline. M6P/IGF2R expression elicited no change in the levels of secreted 52 kDa procathepsin D at 24 hours, 48 hours or 72 hours. In addition to Western blot analyses, we also examined the routing of newly synthesized cathepsin D by metabolically labeling cells with [^{35}S]methionine for 1 hour followed by a 4 hour chase. Figure 11 demonstrates radiolabelled cathepsin D immunoprecipitated from both conditioned media and cell extracts at various time points. Although several isoforms of cathepsin D were observed from cell extracts, no change in the levels of any of these isoforms of cathepsin D was detected in cells expressing or not expressing M6P/IGF2R. Similarly, no change in cathepsin D levels in the conditioned media was detected.

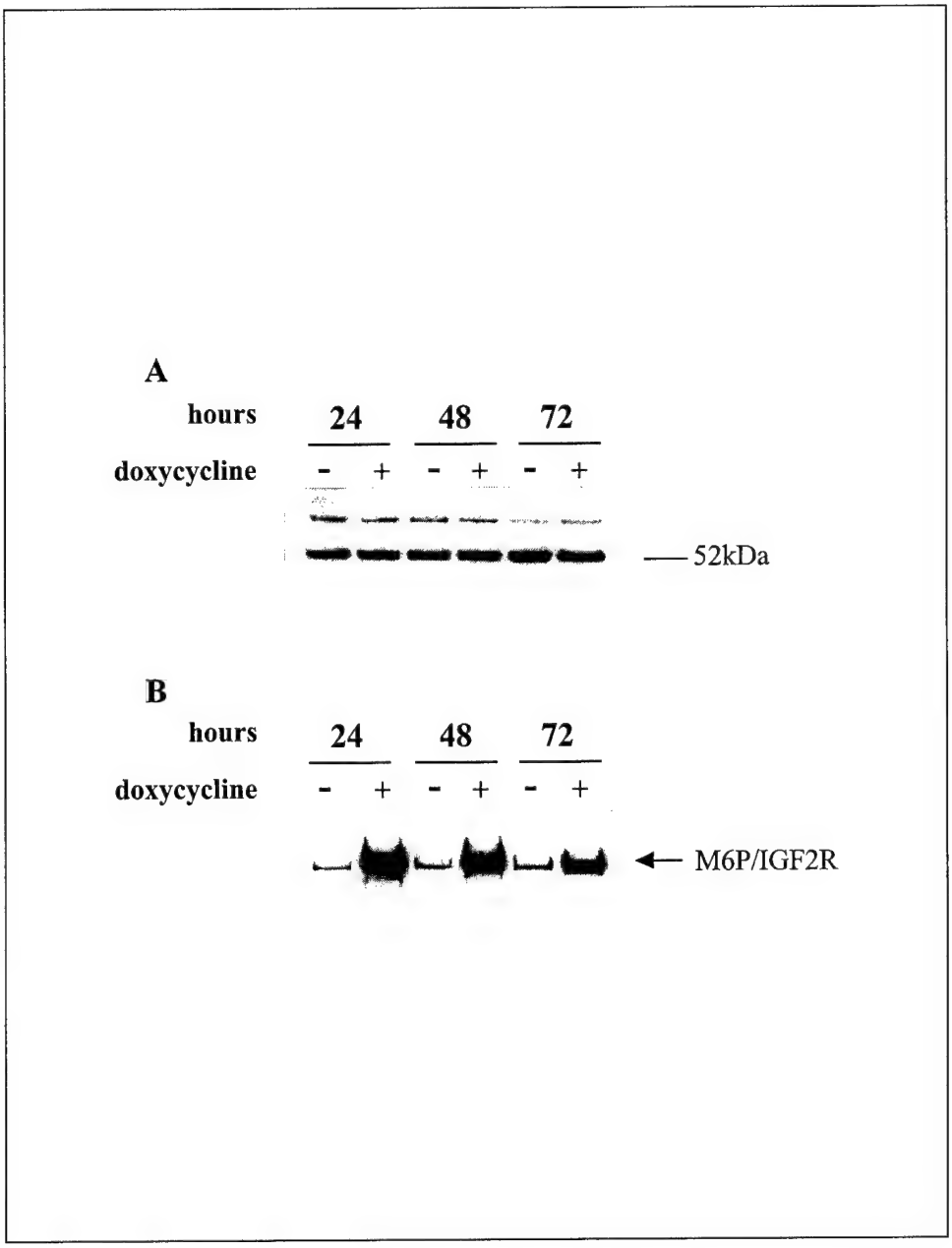

Figure 10. M6P/IGF2R overexpression does not alter cathepsin D secretion. iwt1 cells were treated with or without 1 μg/ml doxycycline in serum free media and the conditioned media collected at various time points, concentrated and analyzed by Western blot for any changes in cathepsin D secretion. Panel **A** shows there was no difference in cathepsin D secretion for any of the time points investigated. Panel **B** shows Western blot analysis of M6P/IGF2R induction with doxycycline treatment at the various time points.

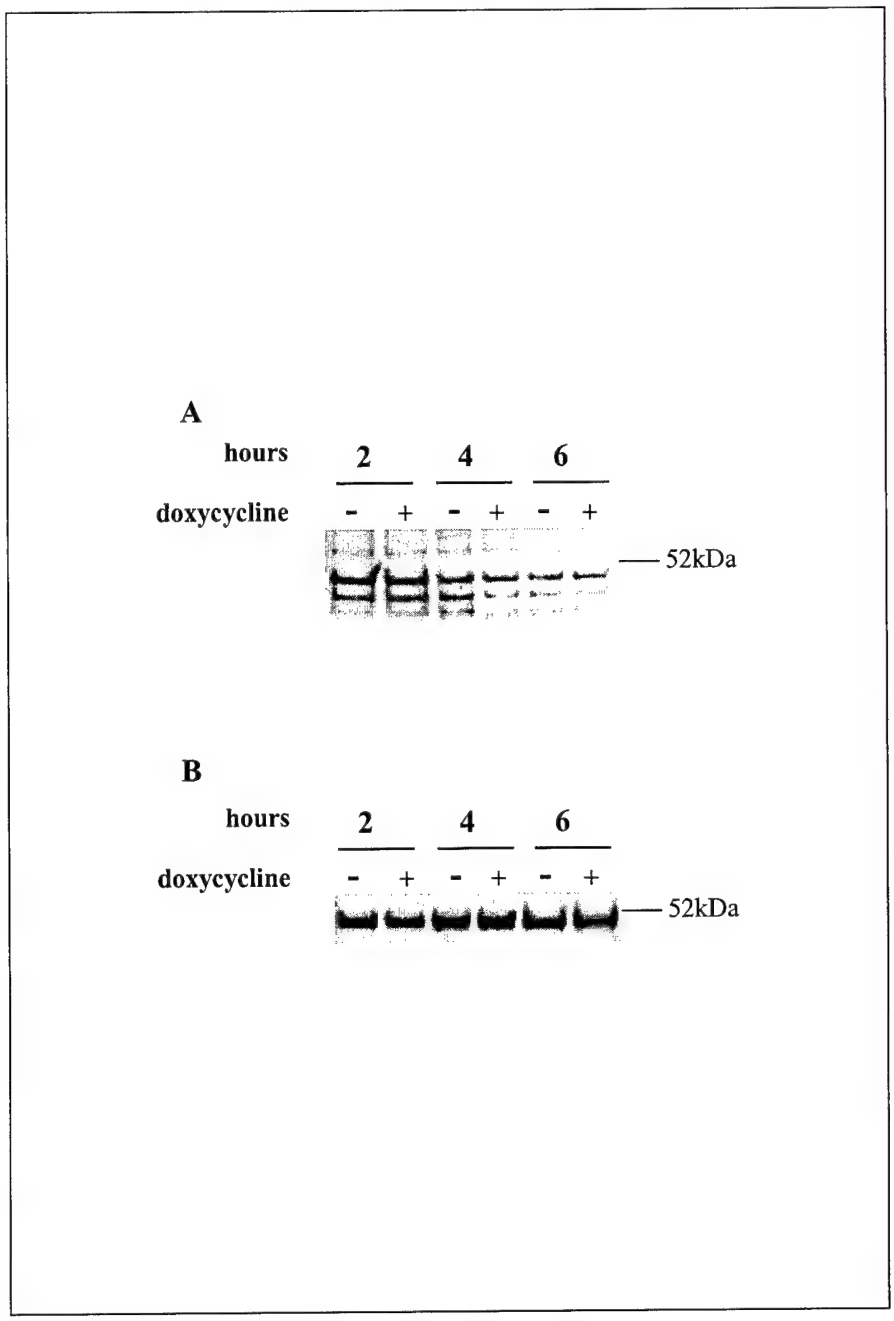

Figure 11. M6P/IGF2R overexpression does not alter routing of cathepsin D. Cells were plated in 6 cm dishes and treated with or without doxycycline for 48 hours to induce M6P/IGF2R expression. The cells were then metabolically labeled (1hr pulse with [^{35}S]methionine then 4 hours chase) and cathepsin D was immunoprecipitated from **A**, cell extracts and **B**, conditioned media. Although several isoforms were detected, there was no difference in cathepsin D secretion at any time point.

Cells overexpressing wt M6P/IGF2R do not appear to internalize IGF2 at an increased rate

It is generally accepted that one function of M6P/IGF2R at the cell surface is to internalize IGF2 target the ligand for lysosomal degradation (29;30). To address whether the induced receptor in the system we employed was capable of this function, we examined the ability of cells overexpressing M6P/IGF2R receptor to internalize IGF2. Cells were plated in 24-well dishes, treated without or with doxycyline for 48 hours and allowed to bind radiolabelled IGF2 at 37°C for 5 minutes, 30 minutes or 60 minutes. The cells were washed to remove any surface bound ^{125}I-IGF2 and lysed. The lysates were assessed with a gamma counter to determine levels of internalized radiolabelled IGF2. We predicted that cells overexpressing the receptor would result in higher levels of internalized IGF2 because of greater numbers of M6P/IGF2R cycling from the cell surface to intracellular compartments. However, we detected no change in ^{125}I-IGF2 internalization in cells overexpressing the receptor compared to controls at any time point (Figure 12).

IGF2 binds to microsomal membrane preparations but not the cell surface of iwt1 cells

Since we saw no evidence of endocytosis of IGF2, we wanted to determine whether transfected M6P/IGF2R was capable of binding IGF2. We performed radioligand binding assays. Membrane preparations of iwt1 cells treated without or with 1 μg/ml doxycyline for 24 hours were isolated, dried onto 96-well dishes and allowed to bind ^{125}I-IGF2 with various concentrations of competing unlabelled IGF2. The amount of bound radiolabelled IGF2 after washing was determined by counting each well with a gamma counter. Membrane preparations from cells treated with doxycycline to overexpress M6P/IGF2R showed at least a three-fold increase in ^{125}I-IGF2 binding above that of control cells indicating that transfected M6P/IGF2R could indeed bind IGF2. Figure 13 is representative of two sets of experiments with triplicate values for each point.

It was curious to us that with 10- to 15-fold induction of M6P/IGF2R (by densitometry of Western blot analysis), the increase in IGF2 binding of cells treated with doxycycline was only three times that of control cells. However, it has been suggested that M6P/IGF2R acts in dimers and perhaps even tetramers (31). A three-fold increase of IGF2 binding supports this dimerization hypothesis.

Since only 5% to 10% of the cellular pool of M6P/IGF2R is present at the cell surface, we were interested to determine whether surface expression of the induced receptor could be detected and if it was also able to bind IGF2. iwt1 cells were grown on coverslips, treated with doxycyline for 48 hours and stained with primary polyclonal antibody at 4^{0}C for 1 hour. The cells were then fixed, permeablized, incubated with immunofluorescent secondary antibody and viewed by confocal microscopy. Figure 14 A demonstrates surface expression of induced M6P/IGF2R. To assess IGF2 cell surface binding, cells were

plated in 24-well dishes in triplicate, treated without or with doxycycline for 48 hours and allowed to bind radiolabelled IGF2 with various competing concentrations of unlabelled ligand for 4 hours at 4^0C. The cells were washed to remove any unbound ligand and then lysed in order to determine the ^{125}I-IGF2 binding with a gamma counter. Figure 14B is representative of 3 experiments and demonstrates no detectable difference in cell surface IGF2 binding of cells overexpressing M6P/IGF2R compared to control cells.

It is possible that although intracellular pools of induced M6P/IGF2R bind IGF2, binding at the cell surface is not evident because of receptor conformation changes when the receptor is exposed to intracellular space. It is also possible that IGF2 binding at the cell surface is not evident in our assays because most of the M6P/IGF2R pool is involved in Golgi-to-lysosomal trafficking and is located within the cell, rather than on the cell surface. We hypothesize that our negative result may imply that M6P/IGF2R targets autocrine rather than paracrine IGF2 for lysosomal degradation. Evidence from our lab suggests that M6P/IGF2R regulates growth of cells secreting their own IGF2. Retrovirus vectors encoding IGF2 with reduced affinity for either IGF1R or to M6P/IGF2R were introduced into MCF7 cells and it was demonstrated that affinity for M6P/IGF2R suppressed autocrine IGF2 activation of IGF1R. Further, extracellular IGF2 accumulation and IGF1R activation was reduced by high affinity of IGF2 for M6P/IGF2R (4).

O'Gorman et al (32) also published evidence that M6P/IGF2R is a tumor suppressor of endogenous IGF2 growth. JEG3, a choriocarcinoma cell line that secretes IGF2, was transfected with an antisense M6P/IGF2R cDNA construct. The authors first investigated the *in vitro* growth rate of antisense M6P/IGF2R cDNA transfected cells plated in 10% FBS and demonstrated an increase in growth rate of cells that had reduced M6P/IGF2R levels compared to sense transfected controls. The cell lines were also tested for tumorigeneic growth *in vivo* and tumors from antisense transfected cells were at least 30% larger than tumors from control cells.

In light of data discussed above and the negative data presented in the previous chapter, we revised our previous hypothesis of investigating exogenous IGF2-dependent growth to investigating the growth regulation of cells with endogenous IGF2. Our revised hypothesis therefore states that M6P/IGF2R may operate to inhibit autocrine IGF2 but not efficiently suppress IGF2 after release from the cell. Thus, M6P/IGF2R may function as a growth suppressor in cells that express IGF2. The effort to investigate this revised hypothesis was the focus of the final year of this grant.

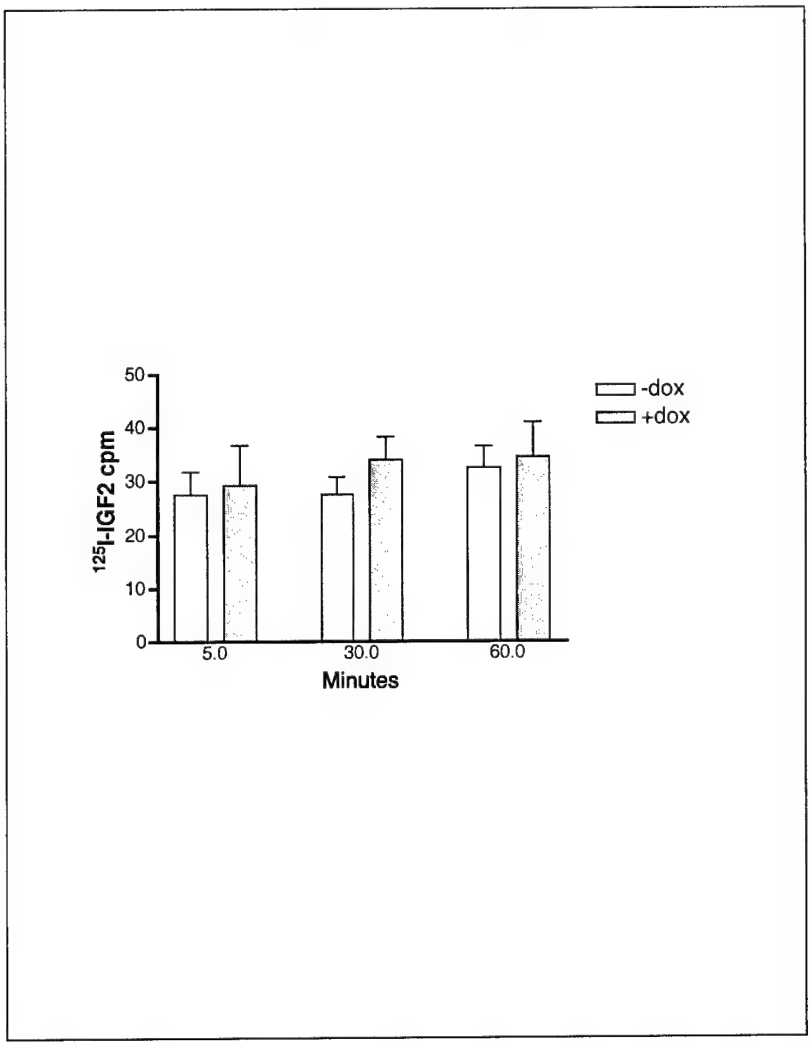

Figure 12. Cells overexpressing M6P/IGF2R do not show an increased rate of IGF2 internalization. 2.5nM ^{125}I-IGF2 was added to iwt1 cells and treated with or without 1 μg/ml doxycycline in serum free media at 37^{0}C with 5% CO_2 in a humidified incubator for the time points indicated. The cells were washed to remove any surface bound radiolabeled IGF2 and then lysed and the amount of internalized radiolabeled IGF2 determined by a gamma counter. There was no significant difference in cells overexpressing M6P/IGF2R compared to cells that were not induced to overexpress the receptor.

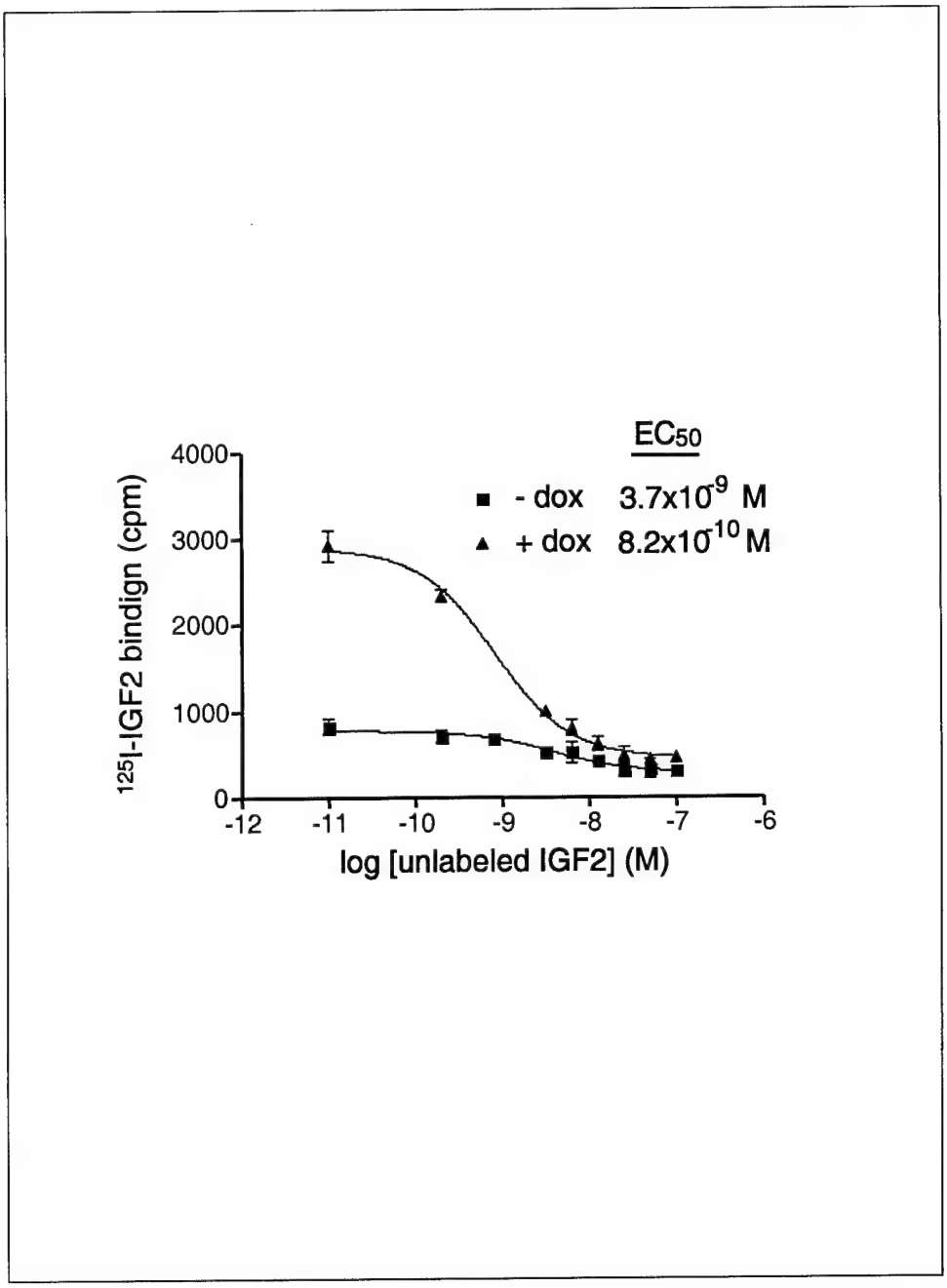

Figure 13. Analysis of radioligand binding assay. The amount of ^{125}I-IGF2 binding to membrane preparations of cells treated with or without 1 μg/ml doxycycline was determined for triplicate samples. Lysates of cells overexpressing M6P/IGF2R demonstrated higher IGF2 binding.

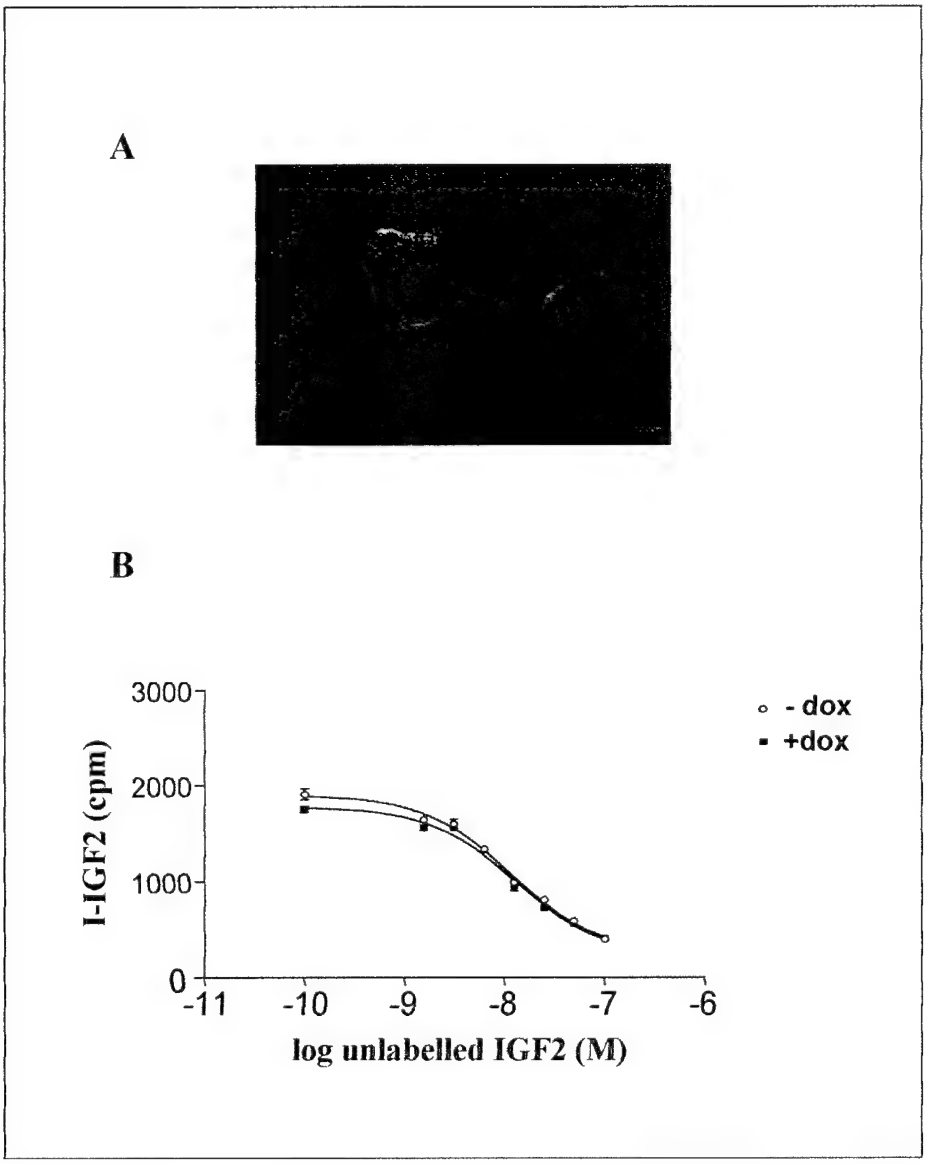

Figure 14. IGF2 binding to the surface of iwt1 cells. **A**, surface staining of live cells with primary antibody at 4^0C demonstrated that overexpressed M6P/IGF2R could be found at the surface of doxycycline induced cells. **B**, iwt1 cells were treated for 24 hours without or with 1 μg/ml doxycycline in serum free media. Radiolabeled IGF2 was allowed to bind at 4^0C for 4 hours with various concentrations of competing unlabelled IGF2. The cells were washed to remove unbound IGF2 and then lysed to determine the amount of bound radiolabeled IGF2. There was no difference in the level of IGF2 bound to the surface of cells expressing M6P/IGF2R receptor compared with cells not expressing the receptor. Each data point was done in triplicate and the graph is representative of 3 experiments.

Identification of IGF2 secreting clones

iwt1 cells were transfected with an IGF2 expression vector (4), grown in selective medium for 5 weeks and single clone populations isolated and expanded. IGF2 expressing clones were identified by dot blot assay on aliquots of serum-free conditioned media (Figure 15). From 45 single-clone populations expanded, two were chosen (iwt1IGF2 11 and iwt1IGF2 44) for studies that examined the effect of M6P/IGF2R overexpression endogenous IGF2-dependent growth.

Immunofluorescene of IGF2 secreting cells

We initially performed double labeling experiments to determine the intracellular localization of IGF2 compared to M6P/IGF2R. We postulated intracellular interaction between IGF2 and M6P/IGF2R since IGF2 is glycosylated and travels through the Golgi apparatus where there would be possibility for interaction with M6P/IGF2R at the trans Golgi network. We hypothesized that interaction between these two proteins may result in targeting of IGF2 for lysosomal degradation before the ligand is secreted and available for activation of IGF1R. Figure 16 shows confocal microscopy images of iwt1IGF2 11 cells that were dual stained for IGF2 and M6P/IGF2R. Figure 16B shows the entire field of cells that were stained, panel A shows M6P/IGF2R immunostaining, panel C shows IGF2 staining and panel D is a merge of M6P/IGF2R and IGF2 staining. The staining pattern of IGF2 was similar to published images (33) and also localized to compartments that overlapped with staining for M6P/IGF2R. This colocalization suggests interaction between the two proteins. Figure 16E and F are images of iwt1 cells without doxycycline treatment stained for either M6P/IG2R or IGF2. These images demonstrate that there is no background of endogenous IGF2 in the parental iwt1 cell line and in the absence of doxycycline treatment, M6P/IGF2R is still not expressed.

Proliferation assays

We performed WST-1 proliferation assays to study the effect of induced overexpressed M6P/IGF2R on the growth rate of cells secreting IGF2 compared to the parental cell line. iwt1IGF2 cells and parental iwt1 cells were plated in 96-well dishes and treated with or without doxycycline to induce M6P/IGF2R expression for 24 hours. Growth was determined by WST1 assay at the time points indicated. Although a growth advantage due to endogenous IGF2 was evident, these assays were difficult to interpret primarily because the cells would die before the end of the assay. This is perhaps a result of selecting clonal populations from cells which are more sensitive to lack of serum. Generally, cells were able to survive up to day 4 in the 96-well dish assays. In at least 3 assays (Figure 17A), we were unable to determine any effect of M6P/IGF2R overexpression on endogenous IGF2 stimulated growth when the

cells were plated in a final concentration of 0.5% FBS. However, we were able to repeat (two times) results of M6P/IGF2R induced growth suppression when cells were stripped in serum free media for 24 hours and then plated in charcoal stripped serum (CCS) or CCS supplemented with 1 nM estrogen (Figure 17B). It must be noted that this difference in growth rate was only observed at or beyond day 4 of the assay suggesting perhaps that under certain IGF2 dependent growth environments, M6P/IGF2R has the ability to regulate growth.

We also investigated anchorage independent growth with the autocrine IGF2 model system. Cells were plated in soft agar with 0.5%FBS and colonies allowed to grow for 10 to 14 days. Our results show that although there is some growth advantage of cells expressing IGF2 compared to the parental cell line, colony formation was still unaffected by overexpression of M6P/IGF2R. Figure 16C shows that iwt1IGF2 44 treated with doxycycline to induce M6P/IGF2R overexpression showed no retarded colony growth compared to control cells and is representative of 2 experiments.

Kinetics of IRS-1 phosphorylation remain unchanged

Our hypothesis predicted that overexpression of wt M6P/IGF2R in IGF2 secreting cells would result in increased degradation of intracellular IGF2 and thus prevent IGF2 secretion and IGR1R activation. We again examined IRS-1 phosphorylation as a measure of IGF1R activation. iwt1IGF2 cells were plated in 6cm dishes and treated with or without doxycycline in growth media for 48 hours. After serum starvation for 24 hours, the serum free media was replaced for the time points indicated in Figure 26 after which the cells were lysed and cell extracts subjected to immunoprecipitation (IP) with anti-IRS-1 antibody followed by Western blotting with an anti-phosphotyrosine monoclonal anitbody. Figure 18A is an IP/Western blot of IRS1 phosphorylation kinetics of uninduced iwt1 treated with 40nM IGF2 for 5 minutes with or without induction of M6P/IGF2R compared to IRS1 phosphorylation profile of iwt1IGF2 cells that secrete IGF2. It is clear from these experiments that IRS1 is phosphorylated above the levels of the serum starved iwt1 parental cells suggesting that endogenous levels of IGF2 results in activation of IRS-1. However, Figure 18B also demonstrates that IRS-1 phosphorylation is not reduced due to M6P/IGF2R overexpression.

M6P/IGF2 R overexpression does not affect apoptosis

Both IGF1 and IGF2 have been shown to confer resistance to apoptosis inducing reagents. Our hypothesis predicts that overexpression of M6P/IGF2R would result in increased apoptotic sensitivity due to a reduction in IGF2 available for activation of the anti-apoptotic response. To investigate whether M6P/IGF2R overexpression resulted in increased apoptosis, iwt1 cells and iwt1IGF2 cells were plated in

96-well dishes in triplicate and treated with or without 40 nM IGF2, 500 ng/ml doxycycline and 5 μg/ml cisplatin for up to 4 days. WST1 readings were obtained at time points indicated in Figure 19. Our data shows that M6P/IGF2R does not sensitize cells to cisplatin-induced apoptosis.

We also considered investigating the kinetics of Akt phosphorylation since it has been shown that Akt phosphorylation plays an important role in the antiapoptotic response of IGF2 through IGF1R activation (34). However, these clones of MCF7 cells produce barely detectable levels of Akt and phosphorylated Akt was undetectable by Western blotting (data not shown).

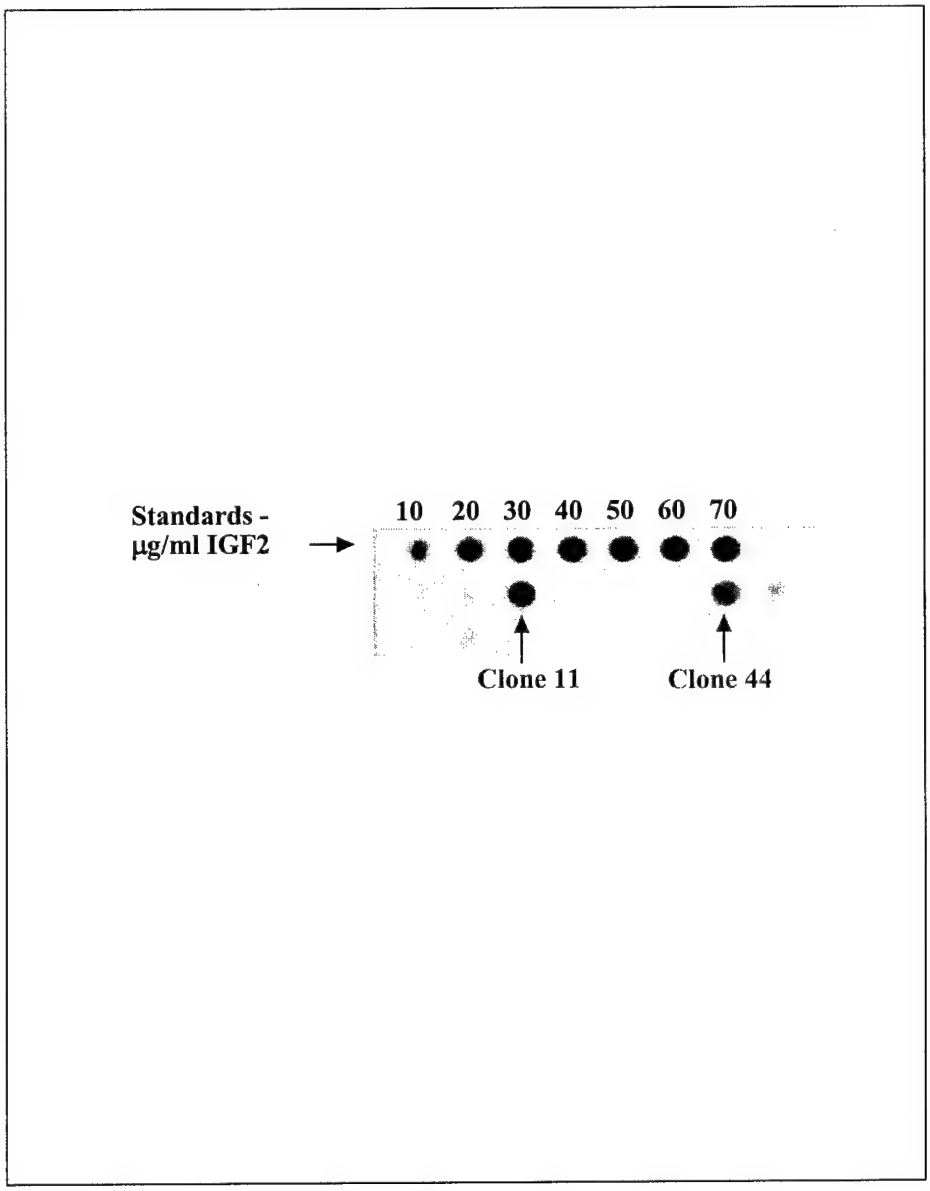

Figure 15. Screening of cells expressing IGF2. Conditioned media from single cell clones of iwt1 cells transfected to constitutively express IGF2 was analyzed by dot blot with antibody against human IGF2. The figure shows analysis of conditioned media (250 µl of 500 µl) from 12 clones grown to approximately 80% confluence in 24-well dishes. Two clones, iwt1IGF2 11 and iwt1IGF2 44, were further expanded because the expressed higher levels of IGF2.

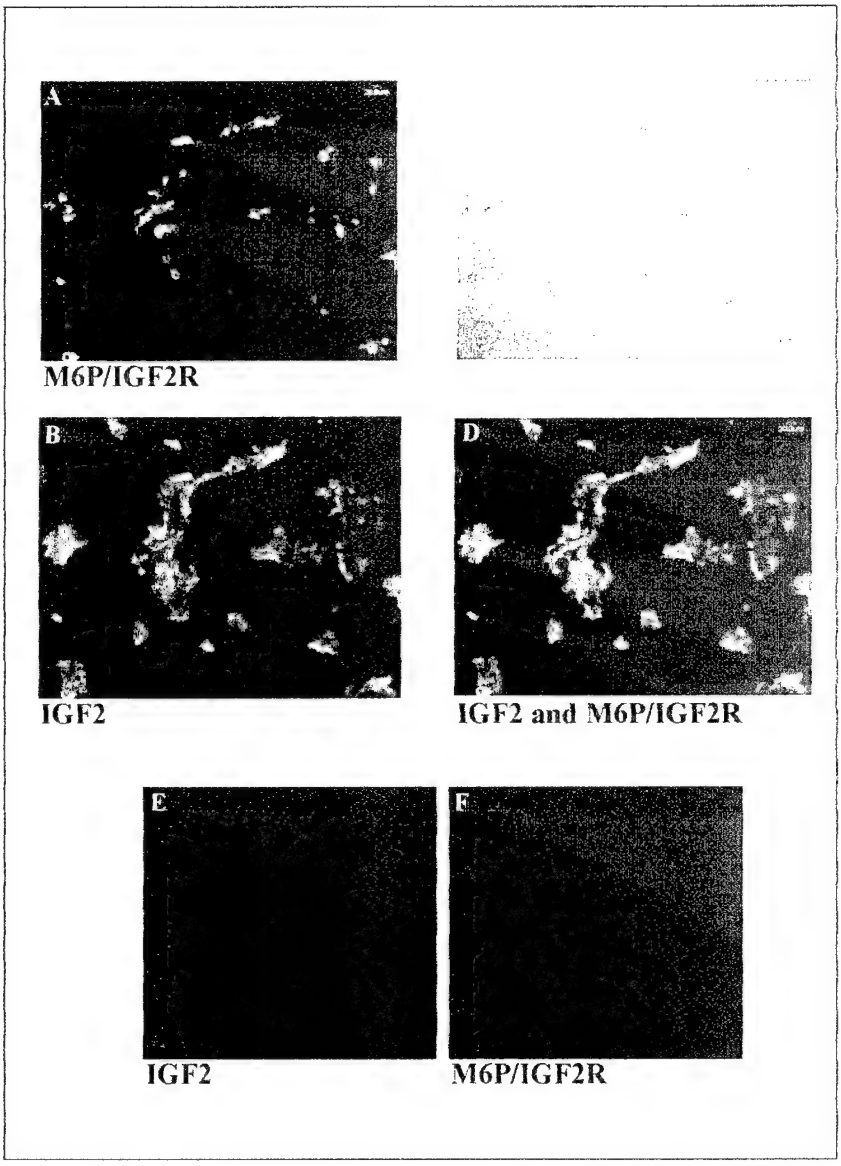

Figure 16. Immunocytochemistry demonstrates localization of IGF2 and M6P/IGF2R in iwt1IGF2 cells. iwt1IGF2 44 cells (**A**, **B**, **C**, **D**) were plated on coverslips in 10% FBS for 24 hours then treated with 500ng/ml doxycycline in growth media for 24 hours. The cells were fixed, permeablized and double labeled for **A**, M6P/IGF2R and **B**, IGF2. Panel **D** shows that areas of IGF2 and M6P/IGF2R overlap. **C** shows the entire field of cells stained. The parental iwt1 cell line, not treated with doxycycline, was also stained for IGF2 and M6P/IGF2R, **E** and **F**. Mag. X200. Note there is no expression of either protein in the uninduced iwt1.

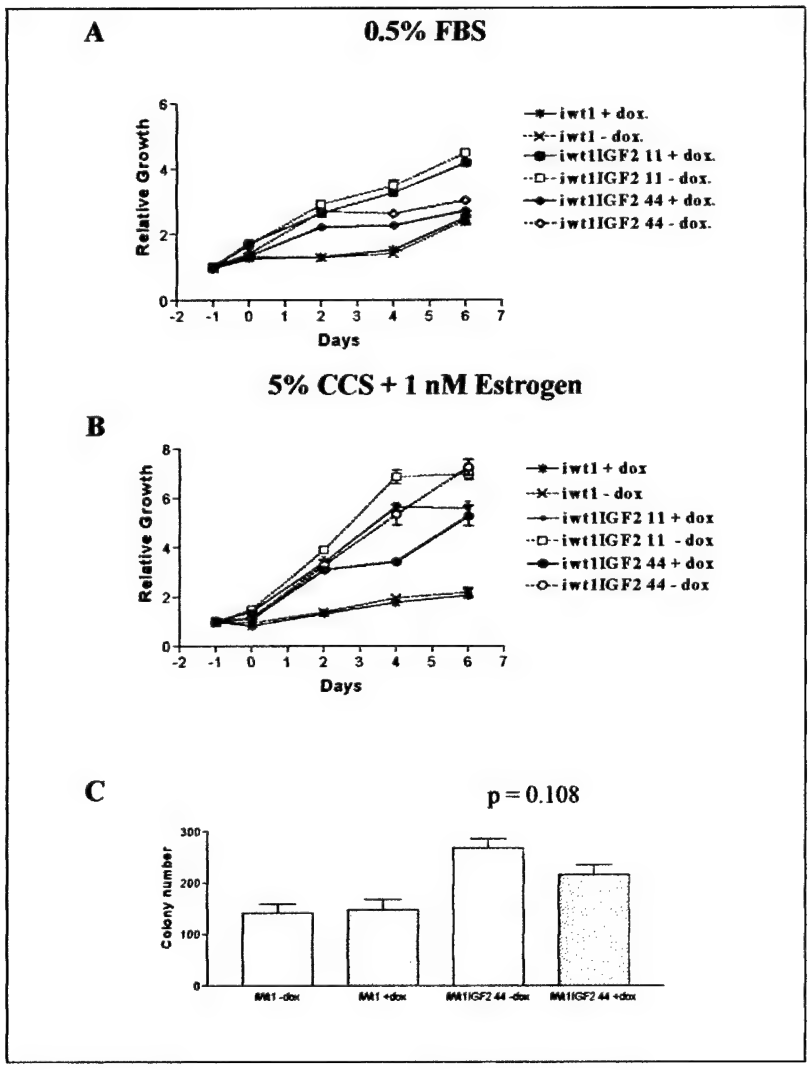

Figure 17. Proliferation of iwt1IGF2 cells. iwt1IGF2 cells were plated in **A**, 5% CCS with 1nM estrogen or **B**, 0.5% FBS, in triplicate in 96-well dishes and treated with or without doxycycline to induce wt M6P/IGF2R expression. Proliferation was determined by WST-1 at the times indicated. The graphs are representative of at least 2 experiments and the error bars represent the SEM of data points in triplicate. **C,** iwt1IGF2 44 cells and parental iwt1 cells were plated in soft agar in 0.5% FBS and treated with or without doxycycline. The number of colonies was determined with an automated colony counter after 14 days. There is no significant difference between cells treated with or without doxycycline. This experiment is representative of 2 experiments of both iwt1IGF2 11 and iwt1IGF2 44.

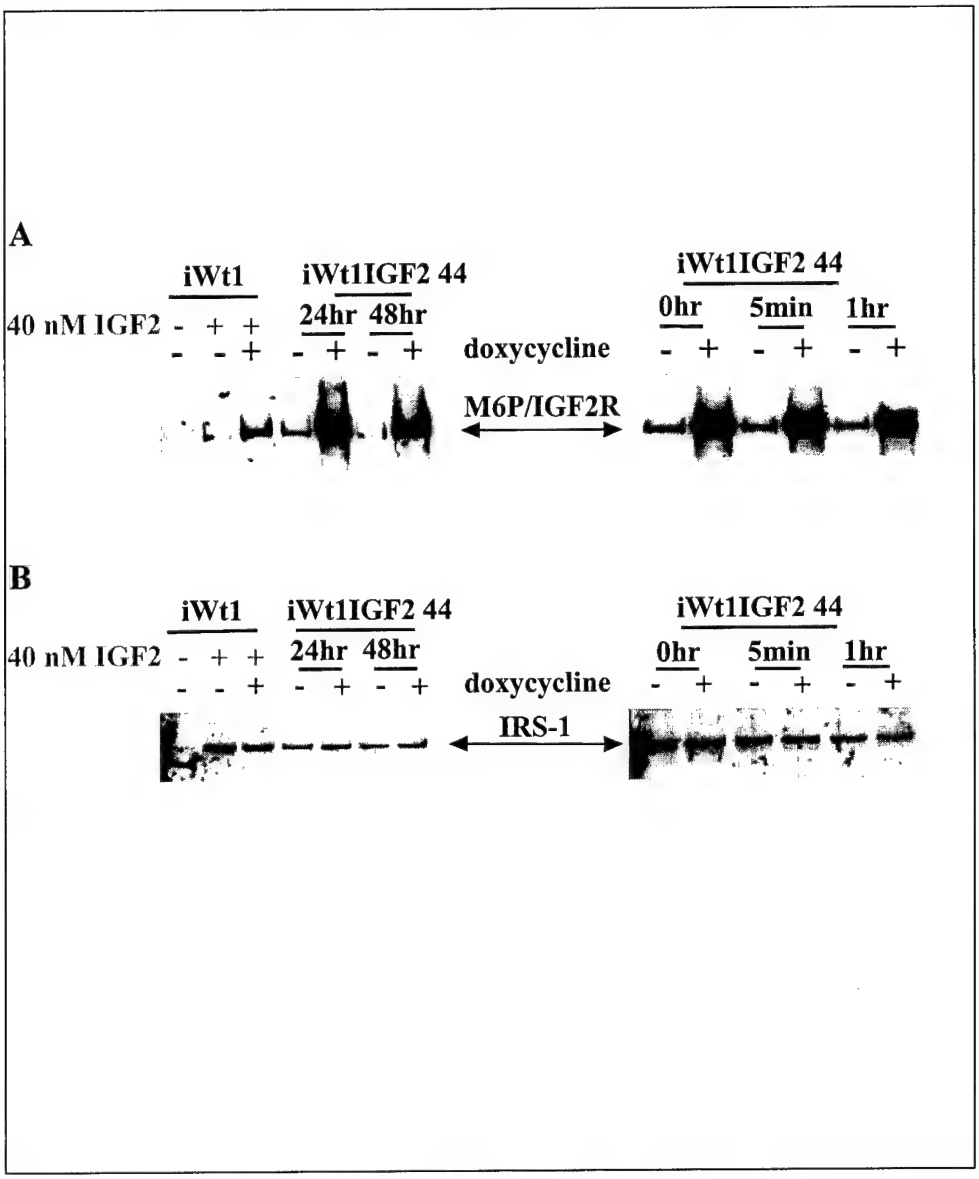

Figure 18. IRS-1 Phosphorylation Kinetics. iwt1IGF2 44 cells were treated with serum free media for the times indicated. Lysates were collected, immunoprecipitated with IRS-1 antibody and immunoblotted with a phosphotyrosine antibody. Western blot analysis of pre-immunoprecipitated lysates was also performed to demonstrate M6P/IGF2R induction. Representative gels of three independent experiments are shown. There was no significant difference in IRS-1 phosphorylation at any time point investigated. Parental iwt1 cells were included in this experiment to demonstrate basal IRS-1 phosphorylation levels when cells are serum starved compared to levels with IGF2 stimulation.

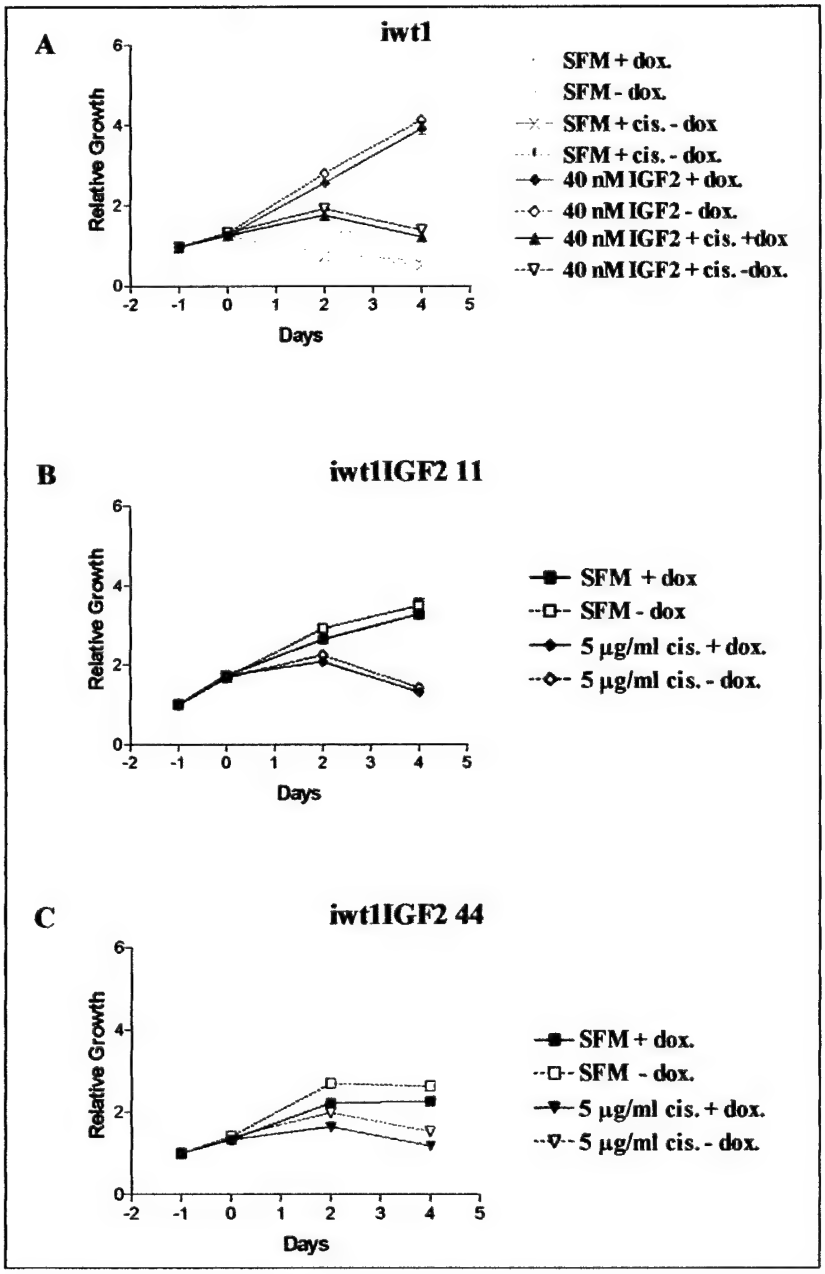

Figure 19. Determination of viable cells after cisplatin-induced apoptosis. **A**, iwt1 cells, **B**, iwt1IGF2 11 cells and **C**, iwt1IGF2 44 cells were plated in 96-well dishes and treated with or without 500 ng/ml doxycycline for 24 hours. The cells were then treated on Day 0 with or without 40 nM IGF2 for 1 hour before adding cisplatin to a final concentration of 5 µg/ml. Cell viability was assessed by WST1 at the time points indicated.

KEY RESEARCH ACCOMPLISHMENTS

1. MCF7 clones that inducibly express wildtype M6P/IGF2R and mutant M6P/IGF2R constructs.
2. MCF7 clones that inducibly express wildtype M6P/IGF2R and constitutively express IGF2.
3. Demonstration that M6P/IGF2R is an inefficient antagonist of IGF2 dependent growth.

REPORTABLE OUTCOMES

I. PhD degree was obtained by Stacey DaCosta under support of this grant.

II. Publications:

1. **Stacey A DaCosta**, Lisa M Schumaker, Adam J Oates, Kevin J Cullen and Matthew J Ellis. Mannose 6-Phosphate/Insulin-like Growth Factor 2 Receptor (M6P/IGF2R) is an Inefficient Antagonist of Extracellular IGF2. (Manuscript in Preparation.)
2. **Stacey A DaCosta**, Lisa M Schumaker and Matthew J Ellis. Mannose 6-Phosphate/Insulin-like Growth Factor 2 Receptor, a *Bona Fide* Tumor Suppressor or Just a Promising Candidate? *Journal of Mammary Gland Biology and Neoplasia* 5(1):85-94, 2000.
3. Adam J Oates, Lisa M Schumaker, Sara B Jenkins, Amelia A Pearce, **Stacey A DaCosta**, Banu Arun and Matthew J Ellis. The Mannose 6-Phosphate/Insulin-like Growth Factor 2 Receptor (M6P/IGF2R), A Putative Breast Tumor Suppressor Gene. *Breast Cancer and Research Treatment* 47:269-281, 1998

III. Abstacts:

1. Stacey A DaCosta, Lisa M Schumaker, Adam J Oates, Kevin J Cullen and Matthew J Ellis. Mannose 6-Phosphate/Insulin-like Growth Factor 2 Receptor (M6P/IGF2R): A Putative Tumor Suppressor of IGF2-dependent Growth. American Association of Cancer Research 92nd Annual Meeting, New Orleans, LA Abstract #2129 3/2001
2. Stacey A DaCosta, Lisa M Schumaker, Adam J Oates and Matthew J Ellis. Mannose 6-Phosphate/Insulin-like Growth Factor 2 Receptor (M6P/IGF2R) is an Inefficient Antagonist of Etracellular IGF2. DOD Breast Cancer Research Era of Hope Meeting, Atlanta, GA. Abstract # I-9 6/2000
3. Stacey A DaCosta, Lisa M Schumaker, Adam J Oates and Matthew J Ellis. Mannose 6-Phosphate/Insulin-like Growth Factor 2 Receptor (M6P/IGF2R) is an Inefficient Antagonist of Etracellular IGF2. American Association of Cancer Research 91st Annual Meeting, San Fransisco, CA Abstract #5002 4/2000

4. Stacey A DaCosta, Lisa M Schumaker, Adam J Oates and Matthew J Ellis. Mannose 6-Phosphate/Insulin-like Growth Factor 2 Receptor (M6P/IGF2R) as a tumor suppressor gene. American Association of Cancer Research 89th Annual Meeting, New Orleans, LA Abstract #4230 4/1998

CONCLUSIONS

This investigation focuses on determining whether M6P/IGF2R may act as a tumor suppressor by antagonizing IGF2-dependent proliferation of malignant cells. There are a few previously published studies that report observing a growth regulatory role for M6P/IGF2R. Our laboratory demonstrated that IGF2 with affinity for M6P/IGF2R suppressed autocrine IGF2 activation of IGF1R (4). O'Gorman et al (32) also demonstrated increased *in vitro* and *in vivo* growth rates of the choriocarcinoma cell line, JEG3, transfected with antisense M6P/IGF2R cDNA to reduce the receptor levels. However, in our present studies, we have been unable to support the theory that M6P/IGF2R is a tumor suppressor. Our negative data may be due to one of two possibilities; (1) M6P/IGF2R does not function as a negative growth regulator in breast cancer or, (2) the growth suppressive role of M6P/IGF2R is not relevant in the systems investigated in our studies.

M6P/IGF2R may not be a tumor suppressor gene

Although the genetic data is compelling, it is possible that M6P/IGF2R does not function as a tumor suppressor in any system or at least not in breast tissue. Genetic evidence demonstrates that the *M6P/IGF2R* locus is subject to LOH in breast, liver and lung tumors and is accompanied by loss of function mutations in the remaining allele, a hallmark of tumor suppressor genes. In reviewing the genetic evidence, it must be noted that although LOH at the *M6P/IGF2R* locus has been reported in approximately 30% of breast cancer (8), the sample sizes were small which therefore raises some uncertainty as to the actual frequency of LOH at this locus. Due in part to larger sample sizes, the evidence for the greater frequency of LOH at the *M6P/IGF2R* locus in liver and lung carcinomas is more compelling. However, LOH is insufficient evidence to describe a gene as a tumor suppressor gene since loss of a gene locus does not address whether the deletion resulted in the malignant phenotype or was a result of an already acquired malignant phenotype. Thus the genetic evidence needs to be supported with functional data.

The model systems utilized in this study may not be ideal for investigating the effect of M6P/IGF2R on IGF2-dependent growth

It is important to consider that M6P/IGF2R does indeed play a role in antagonizing IGF2 dependent growth but that our model systems were not suited to investigate this question for a number of reasons. Experiments with D9 cells showed no growth suppressive effects due to M6P/IGF2R re-expression in these cells. However, D9 cells are unsuited for studying the effect of M6P/IGF2R action on

IGF2 dependent growth since we found these cells unresponsive to IGFs. We then utilized the IGF2-sensitive MCF7 cells to investigate the effect of constitutive expression of M6P/IGF2R and various mutant receptor constructs. We saw no change in IGF2 dependent proliferation due to M6P/IGF2R overexpression compared to control cells. To confirm our negative results, we generated an inducible cell line (using the Tet-On system in MCF7 cells) where M6P/IGF2R expression is induced with doxycycline treatment. We were unable to demonstrate any negative growth effects due to M6P/IGF2R overexpression in this inducible system. These negative results and other data led us to hypothesize that M6P/IGF2R may function as an IGF2 antagonist only in cells secreting IGF2. It is possible however, that our inability to detect any consequence of M6P/IGF2R overexpression on exogenous IGF2 dependent growth is due to the fact that the cells utilized for the inducible system were less sensitive to IGFs and required higher levels of IGF2 for a growth response compared to the cells used in the constitutive experiments. Perhaps the greater concentrations of IGF2 ligand needed to effect a growth response was more than the levels of overexpressed M6P/IGF2R could target for degradation and prevent a mitogenic response.

We then transfected the inducible iwt1 cells to constitutively express IGF2 and performed experiments to determine whether M6P/IGF2R could affect proliferation. We were able to detect a growth advantage in cells secreting IGF2 compared to the parental iwt1 cell, but inducible overexpression of M6P/IGF2R had no effect on autocrine IGF2 dependent growth in our assays. Lack of M6P/IGF2R effect on autocrine IGF2 dependent growth may again be due to the decreased IGF2 sensitivity of these cells. Further, it is plausible that there are more cells expressing IGF2 without M6P/IGF2R induction than there are cells coexpressing both the transfected proteins. Thus any effect of M6P/IGF2R on autocrine IGF2 would be difficult to demonstrate. Finally, it must be considered that a breast cancer cell line transfected to express IGF2 not be relevant in studying the question of M6P/IGF2R antagonism of autocrine IGF2 dependent growth. Although IGF2 is often upregulated in breast cancer, the ligand is usually secreted by the stromal tissue rather than the malignant epithelial cells (35;36) unlike in case of sarcomas and colon cancer (37).

The effect of M6P/IGF2R overexpression on IGF2-dependent growth may not be evident in short-term assays

Although most of our proliferation assays in 96-well dishes when cells were plated in low serum produced negative results, we were able to demonstrate modest results of M6P/IGF2R antagonism of autocrine IGF2 dependent growth when cells are plated in CCS and supplemented with 1 nM estrogen. It is not unreasonable to postulate that lack of function of M6P/IGF2R may not be obvious in the short term

assays investigated in our studies. The effect of loss of M6P/IGF2R function may result in a subtle growth advantage of a population of cells over a period of time. These extra cell divisions could create a population of cells with a greater risk of potential further "hits" which result in the malignant phenotype. Thus, perhaps the short-term effect of any growth regulation due to M6P/IGF2R action is not obvious but the consequence of loss of M6P/IGF2R function is a growth advantage over a period of time. Overexpression of M6P/IGF2R may therefore result in only modest growth regulation over a period of time. This may be one reason that strong *in vitro* evidence of M6P/IGF2R negatively affecting proliferation has not been obtained, but there has been some success with xenograft experiments (32;38). Recently, Lee et al presented data showing *in vivo* evidence of a negative growth regulatory function of M6P/IGF2R. M6P/IGF2R expression was reduced in MDA-MB-231 cells by transfection with an antisense cDNA construct. The authors reported no change in growth, secretion of cathepsin D or TGFβ levels *in vitro*, although they did detect a two-fold difference in IGF2 internalization, but only after 5-hour incubations. However, in animal studies, there was a significant difference in the tumors formed from cells with reduced M6P/IGF2R compared to sense transfected control cells. Sense transfected cells formed tumors when injected into mice 44% of the time (31/70) compared with 79% of tumors formed from injected antisense transfected cells (49/62). Tumors of cells with reduced M6P/IGF2R expression also showed a 10-fold increase in wet tumor weight after 12 weeks. Perhaps M6P/IGF2R is a tumor suppressor but growth suppressive effects can only be determined when considering the full capacity of all the functions of the receptor *in vivo*. The list of ligands that interact with M6P/IGF2R is increasing and these may have both direct and indirect effects on growth regulation, which may or may not be a result of IGF2 manipulation.

It is noteworthy to highlight two facts: evidence of M6P/IGF2R affecting growth suppression has been from studies where the (1) receptor levels are reduced in a cell type and (2) when soluble M6P/IGF2R is overexpressed (39;40). Perhaps one reason we were unable to see a result in our system was that the level of M6P/IGF2R action are already maximal in the cell lines used and therefore, overexpression of the receptor cannot further alter growth regulation. Thus, the importance of a malignant cell line that is null for M6P/IGF2R for investigating the question of M6P/IGF2R as a tumor suppressor. The fact that soluble overexpression of M6P/IGF2R also suggests that M6P/IGF2R does not have only a local effect on regulating cell function but may be involved in paracrine regulation as well. This further supports the theory presented above, that M6P/IGF2R may indirectly affect growth regulation and the full capacity of the receptor functions must be considered.

Could M6P/IGF2R antagonize IGF2 stimulation of novel signaling molecules ?

In addition to proliferation assays, we investigated the kinetics of IRS-1 phosphorylation. IGF2 mediates mitogenesis and anti-apoptotic activity through IGF1R-mediated activation of downstream signaling molecules including insulin receptor substrate-1 (IRS-1) (26). Adding complexity to the already complicated network of receptor activation of the IGF system, is evidence that IGF2 activates insulin receptor at physiological concentrations (41;42). There are at least two isoforms of IR, IR-A and IR-B, which occur because of alternate splicing. The former is predominantly expressed in breast cancer cell lines and tumors. In normal breast tissue, the IR autophosphorylation in response to IGF2 was always less than 1% compared to insulin stimulation. However, in 8 breast cancer cell lines examined and all except for 1 of 27 archived tumor samples examined, IGF2 was able to induce autophosphorylation of IR comparable to insulin stimulation (42). There is also evidence that IGF2 interacts with IR/IGF1R hybrids although the significance of these hybrids are not well characterized. Possibly, one reason we were unable to see any change in IRS1 phosphorylation kinetics is that IGF2 activates IR or IGF1R/IR hybrids causing phosphorylation of novel downstream signaling molecules that effect a mitogenic and/or antiapoptotic response.

Future Directions

In order to make the case whether or not M6P/IGF2R is a tumor suppressor, it is imperative that more studies be conducted with various cell lines that both do and do not secrete IGF2 to determine the extent of the consequence of M6P/IGF2R disruption on tumor growth. The inducible system is undoubtedly a valuable tool to utilize in order to investigate the effect of the presence or absence of a particular gene at a certain time. Of course, a malignant cell line that has no functional M6P/IGF2R would be an ideal model system and the identification of such a cell line would offer some validation to the theory of M6P/IGF2R as tumor suppressor. In the absence of such a cell type however, generating a cell system with ribozymes that target and reduce M6P/IGF2R expression under the control of an inducible promoter would be an interesting way of examining the effect of M6P/IGF2R functional loss both *in vitro* and *in vivo*.

Monitoring tumor formation in transgenic *m6p/igf2r* knockout mice would also be invaluable. However, mice null for *m6p/igf2r* die perinatally. Perinatal death would not be problematic if mice heterozygous for the *m6p/igf2r* knock-out allele could be observed, since one would anticipate an increased rate of tumor formation in mice with this genotype as a result of a congenital "first hit". However, the paternal *m6p/igf2r* is imprinted in mice, with the result that mice *naturally* exhibit monoallelic M6P/IGF2R expression from the maternal allele. Because of this mechanism, when a

knockout *m6p/igf2r* allele is inherited through the maternal germ line, the lethal phenotype is observed since the paternal M6P/IGF2R allele is silent (43). Meeting tumor suppressor definitions for imprinted genes is therefore highly problematic. One approach to circumventing the problem of perinatal mortality would be the development of an "inducible" *m6p/igf2r* knock out mouse. In this approach, a gene can be deleted in a tissue after organogenesis has been completed. However, these approaches are complex, and so far an inducible *m6p/igf2r* knockout has not been described.

We conclude by stating that the case for M6P/IGF2R as tumor suppressor gene is unproven. The genetic evidence is strong, but the mutations described do not necessarily contribute to the carcinogenic process, until functional experiments have shown this to be the case. In addition to functional experiments, further mutation screens are justified, and LOH mapping exercises should be completed to exclude the possibility that adjacent tumor suppressor genes in 6q26 contribute to the rate of LOH rate at this locus in some malignancies.

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